STUDIES OF PROTEINS BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

A thesis
submitted for the degree of
Doctor of Philosophy
in the
Australian National University

Bogdan E. Chapman
Department of Chemistry
Australian National University
March, 1972

I certify that this thesis is my own original work, except where due reference is made to the work of others.

B. E. Chapman

2/3/72

ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. J.H. Bradbury, for his valuable guidance throughout the project.

I would like to express my gratitude to the staff of the Chemistry Deptartment, particularly Mr. R.J. Nicholls, for their assistance in numerous ways.

For 100 MHz NMR spectra obtained on Varian Spectrometers, I would like to thank Dr. R. Bramley of the Research School of Chemistry, Dr. R.A. Craig of the University of Melbourne and Dr. S. Johns of the Division of Applied Chemistry C.S.I.R.O. Melbourne.

For 220 MHz NMR spectra, I would like to thank Varian Associates, Palo Alto, California and the Science Research Council, I.C.I. Petrochemical and Polymer Laboratory, Runcorn, Cheshire.

Further specific acknowledgements are given in the text.

TABLE OF CONTENTS

SUMMARY

The rate of exchange of the histidine C-2 proton with a deuteron, in D_2O solutions, has been measured, using NMR spectroscopy, for a number of histidine model compounds under various conditions. The main aim of this work was to see if the rate of exchange could be varied by changes in the environment around the histidine imidazole ring, with the view to applying the knowledge gained in the exchange of the C-2 protons of histidine residues in proteins. It was found that the rate of exchange increased with increasing pK of the imidazole group.

This method of C-2 proton exchange was used to obtain an unambiguous assignment of the histidine C-2 resonances in ribonuclease-A. The result obtained confirmed the assignment given by Meadows et al (1968) who published an assignment for ribonuclease-A based on experiments on the enzyme ribonuclease-S.

NMR spectroscopy was used to study the aggregation of α-chymotrypsin, three modified α-chymotrypsins and trypsin by measuring the width of resonances as a function of pH. It was found that α-chymotrypsin aggregated at acid pH and that histidine 57 was involved in the aggregation reaction. The dipolar contribution to peak widths in the spectra of the above enzymes has been studied, with particular reference to the peak widths of histidine C-2 and methionine $-SCH_3$ resonances.

The denaturation of several proteins by the addition

of various denaturants has been studied by NMR spectroscopy. Denaturation of α-chymotrypsin by urea at pH 3 was found to be a multiple step process with disaggregation occurring at low urea concentrations. The unfolding of trypsin by urea and guanidine.HCl at pH 4 was found to be a single step reaction. It was shown that a residual non-covalent interaction, involving a methionine residue, is present in the unfolded trypsin molecule. The unfolding of S-methyl-methionine 29-ribonuclease-A by formic acid was studied to obtain information about the double methionine peak observed by Bradbury & King (1972) during the unfolding of ribonuclease-A by formic acid. A study was made of the urea denaturation of the Paramecium aurelia surface protein 51-A. The NMR spectra support the model proposed for 51-A protein by Reisner et al (1969c).

CHAPTER 1

General Introduction

A basic problem in biological chemistry is the determination of the molecular structure of enzymatic and structural proteins. Native enzymatic proteins derive their specific chemical properties from the folding of the polypeptide chain that brings reactive functional groups into a particular geometry, and the determination of this structure is essential to develop an understanding of the mechanisms of reactions catalysed by the various enzymes.

Information about the three dimensional structure in the crystalline state has been successfully obtained for a number of proteins by the use of X-ray diffraction (Venkatachalam & Ramachandran, 1969). This method however, is only applicable to the solid state. As most enzymatic proteins exist in solution, methods are needed to derive the same structural information in solution that X-ray diffraction has yielded for the crystalline state. Most physicochemical methods applied to proteins in solution yield information either about the molecule as a whole (e.g. viscometry and ultracentrifugation) or about specific amino acids such as those containing aromatic side chains (e.g. ultraviolet and fluorescence spectroscopy).

Nuclear Magnetic Resonance Spectroscopy appears to have the greatest potential of all spectroscopic methods for the investigation of proteins in solution. ^{1}H spectra

are dependent, in principle, on all the amino acid components of the protein as different amino acids give rise to characteristic peaks in the spectrum. Different regions of the protein molecule can be distinguished in the NMR spectrum depending on the number and distribution of each type of amino acid. Alterations in the position or shape of a peak are often indicative of changes in conformation of a protein and can occur when the native protein is perturbed by substrates, inhibitors or changes in the environment such as pH or the addition of denaturants.

The method suffers from several disadvantages. Protein solutions of high concentration (1-10%) are required to obtain useful spectra and some proteins form aggregates or gels at these concentrations. Peaks from native proteins are usually very broad as they are often composed of closely separated resonances from different amino acids, and the slow molecular motion of proteins in solution decreases the nuclear spin relaxation times of individual amino acid protons, thus increasing the width of resonances from these protons. These problems can be reduced by the use of high-frequency spectrometers, as the position of a resonance from an individual amino acid proton depends on the irradiation frequency, and small differences in position will become larger at higher frequencies. Lower concentrations can also be used with high-frequency spectrometers as, in principle, the signal strength is proportional to the square of the radio-frequency field.

Recent reviews on the application of NMR spectroscopy to protein chemistry have been published by McDonald & Phillips (1970) and Roberts & Jardetzky (1970).

A number of different approaches can be used in the application of NMR spectroscopy to protein chemistry. For example, studies of the relaxation rate of the protons of water in enzymatic systems involving a paramagnetic cofactor such as Mn^{2+} have provided a detailed description of the binding of enzyme, cofactor and substrate as well as providing an understanding of the kinetics of the process. A review in this field has been published by Mildvan & Cohn (1970). Another approach is the study of the NMR spectra of small molecule substrates and inhibitors as they are modified by binding to enzymes. A number of papers in this field have been reviewd by Roberts & Jardetzky (1970).

The most powerful NMR approach to structures and interactions of proteins is a direct examination of the resonances from the protons of the protein itself. Saunders et al (1957) obtained the first NMR spectrum of a protein, native ribonuclease A, using a 40 MHz spectrometer. Four broad peaks were observed which could be assigned to aromatic, α-CH, methylene and methyl protons. Other early workers in this field were Bovey & Tiers (1959), Saunders & Wishnia (1962) and Kowalsky (1962). They obtained the spectra of a number of proteins in the native and denatured state and found, as was hoped, that the spectra of the native forms were different to those of the denatured forms, being influenced by the tertiary structure. Numerous papers on

the subject have since been published.

NMR spectroscopy has been used in the study of the denaturation of proteins by heat and the addition of denaturants such as urea and guanidine hydrochloride to solutions of proteins, as dramatic sharpening of various proton resonance bands takes place upon conformational unfolding. The study of unfolding in proteins is useful as a method of obtaining information about the non-covalent bonding present in native proteins. NMR spectroscopy is very useful in these experiments as the unfolding of a number of amino acid residues can be monitored simultaneously This enables a determination to be made as to whether the transition between the native and denatured state is a single or multiple step process. Bradbury & King (1969) have also shown that the presence of residual non-covalent interactions in the denatured state can be determined by the presence of anomalous peaks in the NMR spectra of denatured proteins.

A great deal of work has also been carried out to assign resonances to individual amino acid residues in proteins. Assignments are necessary so that perturbation of resonances accompaning changes in temperature, pH or with reaction of the protein with substrates, inhibitors or metal ions can be related to particular amino acid residues in the protein. Meadows et al (1968) have published an assignment for all four histidine C-2 resonances in bovine ribonuclease. Assignment of histidine resonances is relatively easy compared to other amino acid

resonances as the histidine C-2 peaks are usually well separated from the rest of the peaks in the spectrum. The overlap of peaks makes it difficult to assign resonances arising from protons on amino acids other than histidine.

Markley et al (1968) and Crespi & Katz (1969) have developed a method of eliminating overlapping resonances by isolating proteins in which most of the amino acid protons have been replaced by deuterons. These proteins give simplified spectra with little overlap of peaks which makes the assignment of resonances a lot easier compared with spectra of fully protonated proteins.

In this thesis the technique of NMR spectroscopy has been applied to susceptible problems in protein chemistry.

CHAPTER 2

THEORY

2 A NMR

An account of the theory and practice of nuclear magnetic resonance spectroscopy has been described in a number of texts (Pople et al., 1959; Emsley et al., 1965; Bovey, 1969).

The nuclei of certain atoms possess spin and associated with it a magnetic moment. If an external magnetic field is applied to a system of nuclei with magnetic moments they will tend to take up specific orientations with respect to the magnetic field. For ^{1}H nuclei whose spin quantum number $I=\frac{1}{2}$ there are two orientations possible. The first in which the magnetic moment is aligned parallel to the magnetic field (low energy state) and the second in which the magnetic moment is aligned antiparallel to the magnetic field (high energy state). When the nuclei are irradiated with energy at the correct frequency, in the radiofrequency range, transitions between the two states occur and this gives rise to the peaks, also known as resonances, in the NMR spectrum.

The frequency at which resonance occurs for a particular proton in a molecule depends on its magnetic or chemical environment in that molecule. Different protons exposed to the same magnetic field will resonate at different frequencies and these frequencies are called the chemical shifts of a proton. The chemical shift of a

peak indicates the type of proton (e.g. aromatic, methylene or methyl) and the area under the peak is proportional to the number of such protons present in the molecule. Chemical shifts are given in units of Hz from a reference peak in the spectrum. As the shift in Hz is linearly proportional to the radiofrequency strength used, the unit δ is often used to adjust the chemical shifts, obtained at various radiofrequencies, to a common value. The units of δ are parts per million (ppm). This means that a δ unit consists of 60 Hz at a radiofrequency of 60 MHz, 100 Hz at 100 MHz and 220 Hz at 220 MHz.

The width of a peak in a protein spectrum is dependent on a number of factors and may be regarded as the sum of several terms all contributing to the width. One of these terms is the width of the corresponding peak in the free amino acid (W_a). The width of this peak is composed of the Heisenberg uncertainty in the frequency, which is dependent on the spin relaxation time, and inhomogeneity in the applied magnetic field. These terms are constant and make no additional contribution to the protein peak width.

The dipole-dipole interaction between neighbouring protons is considered to be the major contributor to the peak width of proton resonances in macromolecules (Kowalsky, 1962; Bloembergen et al., 1948). Kubo & Tomita (1954) have derived an equation to determine the dipole-dipole contribution (W_d) to the peak width:-

$$W_d = \frac{1.41 \times 10^{11}\ \tau_c}{b^6}\left[\frac{3}{5} + \frac{1}{1+\omega^2\tau_c^2} + \frac{0.4}{1+4\omega^2\tau_c^2}\right] \qquad (1)$$

where b is the interproton distance in Angstrom units

$\omega = 2\pi\nu$ (ν is the radiofrequency used e.g. 100 MHz)

and τ_c is the correlation time (sec), i.e. the time required for the molecule to turn through an angle of about one radian.

This equation holds provided that the rotational motion of a pair of protons is isotropic and $W_d\tau_c < 0.01$. The first condition is fulfilled by proteins of an approximate spherical shape and the second holds for small globular proteins in solution, where $W_d < 100$ Hz and $\tau_c < 10^{-7}$ sec.

The correlation time for rotation of a rigid sphere is given by an equation developed by Bloembergen et al., (1948):-

$$\tau_c = \frac{4\pi\eta a^3}{3KT} \qquad (2)$$

where η is the viscosity of the solvent,

a is the radius of the sphere,

K is Boltzmann's constant,

and T is the absolute temperature.

Equation (1) shows that an increase in the correlation time or a decrease in the internuclear distance

would broaden a peak and equation (2) shows that the value of the correlation time may be increased by a increase in the size of a molecule. Changes in peak width can be used to follow the mobility of a specific residue in a protein and the aggregation of rigid proteins.

The width of a resonance can also be increased by an amount W_e if the proton concerned is exchanging between two different magnetic environments A and B for which the chemical shifts are δ_A and δ_B in Hz. Pople et al., (1959) and Emsley et al., (1965) have defined three cases as follows:-

(1) Slow exchange where rate $\ll 2\pi(\delta_A - \delta_B)$. Two separate resonances are observed with chemical shifts δ_A and δ_B. If t_A and t_B are the lifetimes of each state, the additional broadening of each peak will be:-

$$W_e = (\pi t)^{-1} \quad \text{where } t = t_A \text{ or } t_B$$

(2) Fast exchange where rate $\gg 2\pi(\delta_A - \delta_B)$. One resonance is observed which occupies an intermediate position between δ_A and δ_B and may be broadened. The value of the chemical shift is:-

$$\delta = P_A\delta_A + P_B\delta_B$$

where P_A and P_B are the populations in state A and state B and its width is given by:-

$$W = P_AW_A + P_BW_B + 4\pi P_A^2P_B^2(\delta_A - \delta_B)^2(t_A + t_B)$$

where W_A and W_B are the widths in the absence of exchange.

(3) Intermediate exchange where rate $\sim 2\pi(\delta_A - \delta_B)$. At this rate a large amount of broadening occurs. Expressions for position and width are complicated and will not be given here.

The width may increase by a term W_s due to spin rotational broadening. Rotation of a molecule is accompanied by rotation of charges, which gives rise to fluctuating magnetic fields. The interaction of these fields with nuclei may provide a mechanism for relaxation. However Pritchard & Richards (1966) have shown that this contribution is relatively small for protons and may be ignored.

For native proteins broadening often arises from non-equivalence in magnetic environments of otherwise identical protons. An amino acid side-chain occuring in different regions of the protein may experience different magnetic environments, depending on the neighbouring amino acid residues. Any peak arising from these side-chains will consist of a number of separate resonances each with a slightly different chemical shift. The increase in width due to this effect may be called W_c.

Broadening may also arise from the presence of paramagnetic nuclei, or nuclei with large quadrupolar moments. However, for protons, broadening arising from magnetic anisotropy is negligible (Gutowsky & Woessner, 1956).

Thus for diamagnetic proteins the width of a

resonance may be written as:-

$$W = W_a + W_d + W_e + W_c$$

2 B Denaturation

A protein is denatured when a major change occurs in the native conformation without the cleavage of of any covalent bond (Tanford, 1968). A general equation for the denaturation of a protein is:-

$$N \rightleftharpoons U \rightleftharpoons D$$

where the native protein N goes to an unfolded state U which may undergo further change to give the final denatured product D. In some cases transitions may be irreversible.

The unfolded state U may be a random coil in which rotation about individual bonds is as unrestricted as rotation about similar bonds in small molecules. In this state a protein is completely unfolded as there are no fixed non-covalent interactions. However, U could also be a partially unfolded state in which fixed non-covalent interactions occur. The state D could be an aggregate, gel or non-native refolded conformation. Often denaturation does not proceed past the unfolded state U.

NMR spectroscopy can be used in the study of denaturation as, on unfolding, the increase in mobility of groups, due to the breakup of non-covalent interactions, causes a decrease in the correlation time of protons in these groups and thus decreases the width and increases

the height of peaks arising from these protons.

The extent of unfolding is defined by the following equation:-

$$F = \frac{h - h_N}{h_U - h_N} \qquad (4)$$

where F is the extent of unfolding,

h is the peak height at a particular level of denaturant,

h_N is the peak height in the native protein

and h_U is the maximum peak height reached in the series. All heights are corrected for dilution effects accompanying the addition of denaturant.

The significance of F depends on the nature of the unfolding. If the transition $N \rightleftharpoons U$ is a single step transition with no intermediates present, and is slow enough for exchange broadening W_e to be negligible, then

$$h = \alpha h_U + (1 - \alpha) h_N$$

where α is the fraction of unfolded molecules. This equation rearranges to give

$$\alpha = \frac{h - h_N}{h_U - h_N} = F \qquad (5)$$

In this case the extent of unfolding is equal to the fraction of unfolded molecules.

If exchange between two states is rapid, exchange broadening occurs, and the above relationship between F and α does not hold. Useing the equation for rapid exchange broadening (NMR section), one can write:-

$$W = \alpha W_U + (1 - \alpha) W_N$$

where W is the peak width at half-height at a particular

concentration of denaturant,

W_N is the peak width at half-height in the native protein

and W_U is the minimum peak width at half-height measured in the series.

For a Lorentzian peak, $hW = h_N W_N = h_U W_U$. Thus

$$\frac{1}{h} = \frac{\alpha}{h_U} + \frac{(1 - \alpha)}{h_N}$$

$$\alpha = \frac{h_U}{h} \frac{(h-h_N)}{(h_U-h_N)}$$

$$\alpha = \frac{h_U F}{h} \qquad (6)$$

The fraction of unfolded molecules, α, is greater than the extent of unfolding, F, throughout the transition.

If there are intermediate configurations present during the transition, equation (5) does not hold. Intermediate configurations may be recognised by the appearance of additional peaks during the transition, or by the non-equivalence of F values among peaks.

CHAPTER 3

EXPERIMENTAL

3 A PROTEINS

Commercial samples of proteins were obtained from the following sources:-

Worthington: Ribonuclease A (phosphate free, lyophilised powder)

α-chymotrypsin (3X crystallized)

Trypsin (2X crystallized)

HEW lysozyme (2X crystallized)

Sigma: Ribonuclease A (5X crystallized)

King (1970) has shown that Sigma ribonuclease contains about 5 moles of sulphate per mole of ribonuclease. The Worthington product is salt free as it has been desalted by the method of Crestfield et al (1963). Most of the other enzymes contained acetate salts and these were removed by dialysis against distilled water.

A small quantity of water soluble surface protein, (immobilisation antigen 51A), from the ciliated protozoan Paramecium aurelia was supplied by Dr. A. Reisner, Division of Animal Genetics, C.S.I.R.O., Epping N.S.W.

3 B MODIFIED PROTEINS

A number of selectively modified proteins were prepared.

Diisopropylphosphate-serine 195- α -chymotrypsin (DFP)

The method of Jansen et al (1949) was used to prepare samples of the inhibited enzyme. 0.5 g. of

α-chymotrypsin was dissolved in 50 ml. of 0.1 M phosphate buffer pH 7.7. To this was added 1 ml. of a solution of 0.1 ml. diisopropylfluorophosphate in 10 ml. of isopropanol, to give a mole ratio of inhibitor to enzyme of 10:1. The solution was stirred for twenty minutes at room temperature, dialysed for 24 hours against 10^{-4} M HCl and the product was isolated by lyophilisation.

3-N-Methyl-histidine 57- α -chymotrypsin (NMH)

The method of Bell (1928) was used to prepare p-nitrophenylsulphonyl chloride. This compound was treated with sodium methoxide (Morgan & Cretcher, 1948) to give the inhibitor p-nitrophenylsulfonate methyl ester. 0.25 g. of the enzyme was dissolved in 30 ml. of 0.1 M phosphate buffer pH 7.9. To this solution 0.035 g. of the methyl ester in 4 ml. of acetonitrile was added over six hours to give an enzyme to inhibitor mole ratio of 1:16 (Nakagawa & Bender, 1970). The solution was dialysed against 10^{-4} M HCl at 4^{o}C for 24 hours and the product was isolated by lyophilisation.

p-fluorophenylsulphonyl-serine 195- α -chymotrypsin (FPS)

The method used was developed by Kallos et al (1963) for the inhibition of the enzyme by p-toluenesulphonyl chloride. 0.50 g. of the enzyme was dissolved in 100 ml. of 0.05 M phosphate buffer pH 7.5. To this solution was added 0.095 g. of p-fluorophenylsulphonyl chloride in 10 ml. of acetonitrile at 0^{o}C, giving a mole ratio of enzyme to inhibitor of 1:20. The acetonitrile solution

was added in two lots over 15 minutes and the solution was stirred for 1 hour. The solution was dialysed against 10^{-4} M HCl at 4^{o}C for 24 hours and the product was isolated by lyophilisation.

The three modified ∝-chymotrypsin compounds were dissolved in d-trifluoroacetic acid and their spectra were obtained and compared with the spectrum of the native enzyme in the same solvent. The spectra of the NMH and FPS compounds had additional peaks present with chemical shifts identical to those of the small molecule model compounds 3-N-methyl-L-histidine and p-fluorophenylsulphonyl chloride respectively. No additional peaks were observed in the spectrum of the DFP compound, as the diisopropyl resonances would occur in the ∝-CH and methyl resonance bands. However, as this reaction is extremely vigorous (Jansen et al., 1949), it is highly unlikely that the modification has not taken place.

S-methyl-methionine 29-ribonuclease A

The method of Link & Stark (1968) was used to prepare this compound. 200 mg. of Worthington ribonuclease was dissolved in 50 ml. of water and the pH was adjusted to 2.5. The solution was saturated with methyl iodide and stirred for 16 hours, at 20^{o}C, in the absence of light. The solution was dialysed against 10^{-4} M HCl at 4^{o}C for 24 hours and the product was isolated by lyophilisation. NMR measurements on the spectrum of the sample showed that the equivalent of one methionine residue had been methylated.

Comments on the specificity of the enzyme modification reactions are given in Appendix IIB.

3 C OTHER MATERIALS

The following compounds were used in deuteration studies:-

L-histidine and β-alanyl-L-histidine obtained from Nutritional Biochemicals Corp. Imidazole and L-histidyl-glycine.HCl from Mann Research Laboratories, and N-acetyl-L-histidine.H_2O from Cyclo Chemical Corp. These compounds were used without further purification.

Urea was recrystallized from water to remove an impurity giving rise to a peak at 8.95 δ. A mixture of HCl and DCL was obtained by diluting 12 M HCl with an equal volume of D_2O. A solution of NaOD was prepared by reacting sodium with D_2O. Other reagents were the best available and were used without further purification.

3 D SPECTROSCOPY

Most of the spectra were obtained on a Perkin-Elmer R-10 60 MHz spectrometer at the Chemistry Department, School of General Studies. 60 MHz spectra were run at 33.4°C using a radiofrequency input of 1mV and a sweep rate of 6.4 Hz per second. 100 MHz spectra were obtained on Varian instruments at the Research School of Chemistry, the Department of Chemistry, Melbourne University and the Division of Applied Chemistry, C.S.I.R.O., Melbourne. Towards the end of the project a 100 MHz JEOL Minimar spectrometer at the Department of Chemistry, School of General Studies, was also used. A few spectra were obtained on 220 MHz Varian spectrometers at the I.C.I. Petrochemical and Polymer Laboratory, Runcorn, Cheshire, and at Varian Associates, Palo Alto, California. These spectra were

obtained by Dr. J. H. Bradbury.

A Digital Equipment PDP-8/S computer was used on-line with the Perkin-Elmer 60 MHz spectrometer, and later with the JEOL 100 MHz spectrometer, to increase the signal-to-noise ratio by averaging spectra. The ratio of signal-to-noise increases in proportion to the square root of the number of scans accumulated (e.g. 400 scans improves the signal-to-noise ratio by 20).

Peripheral equipment on the computer included an analogue-to-digital converter, an ASR-33 teletype, a real time clock and a Tektronix storage oscilloscope.

A basic spectrum averaging program was supplied by Digital Equipment. As the Perkin-Elmer spectrometer does not have a locking system and is very sensitive to temperature baseline drift, the program was modified by Dr. N. L. King to control these factors.

To compensate for magnetic field drift, the computer program aligned a reference peak in each scan with the same peak in the first scan. An external reference was always used when obtaining protein spectra in D_2O. This reference consisted of a thin capillary tube containing a solution of tetramethylsilane or dichloroacetic acid in carbon tetrachloride. This technique, although eliminating any interaction between reference and sample, had the disadvantage of decreasing the volume of sample being irradiated, thus decreasing the sensitivity. Chemical shifts are quoted relative to the tetramethylsilane peak, unless otherwise stated.

It was also found to be necessary to compensate for baseline drift as, after a few hours, the spectrum frequently drifted off scale due to the high amplifier gains used. At the end of each scan, the computer delivered a voltage signal, for an appropriate length of time, to an electric motor that adjusted the setting of a potentiometer controlling the baseline level.

The computer was also programmed to reject scans that were unsatisfactory because of poor resolution, large baseline drift or large magnetic field drift. The chemical shifts, heights, widths and areas of peaks could be typed out on the teletype by a subroutine written into the main computer program. Both the average spectrum and the last single scan could be displayed on the oscilloscope while accumulation of data was taking place. The spectra, as well as being plotted out on the NMR recorder, could be punched out on paper tape or listed by the teletype. The spectra stored on punched tape could be read into the computer at any future date. This feature was found to be very useful for obtaining difference spectra. In this technique, the program aligned reference peaks, and then subtracted one spectrum from the other. This method clearly showed slight differences in spectra that were often difficult to detect in the parent spectra as they were obscured by large peaks.

Solutions for NMR studies were usually at 10% concentration (weight/volume) unless otherwise stated.

The pH of the solutions was measured on a Beckman Research pH meter, fitted with microelectrodes to allow volumes of 0.5 ml. to be read. Values quoted in this thesis for protein solutions in D_2O are pH meter readings uncorrected for deuterium isotope effects. Willumsen (1968) has shown that the relationship between pH and pD for protein solutions is not well defined, and Simpson & Kauzmann (1953) have shown that there is some doubt about the significance of pH values in concentrated urea solutions.

CHAPTER 4

THE DEUTERATION OF HISTIDINE

MODEL COMPOUNDS

4 A INTRODUCTION

A technique for simplifying the analysis and assignment of peaks in C-H NMR spectra has recently been introduced and shows considerable potential. This technique involves replacing specific C-H protons in a protein with deuterons. Markley et al (1968) and Crespi & Katz (1969) have published papers on this subject. Markley et al (1968) obtained deuterated amino acids by the hydrolysis of protein isolated from algae grown in D_2O. During the hydrolysis a few proton types were back-exchanged to the protonated form, but most of the amino acids remained fully deuterated.

Partially deuterated staphylococcal nuclease was isolated from the bacterium Staphylococcus aureus by growing the bacterium on the deuterated protein hydrolysate, with an excess of fully protonated tryptophan and methionine. The NMR spectrum was greatly simplified, consisting only of peaks due to tryptophan, methionine and amino acids that had back-exchanged protons for deuterons during the isolation of the deuterated protein hydrolysate.

This method is of limited use and can only be used for proteins isolated from organisms such as bacteria. Another way to obtain partially deuterated proteins is to develop mild chemical methods to exchange specific protons

for deuterons in fully protonated proteins. An example of this is the exchange of the C-2 proton of tryptophan by treatment with d-trifluoroacetic acid (Bak et al., 1967). Norton (1971) has shown that the C-2 protons of tryptophan residues in lysozyme may also be exchanged by the same method without irreversibly denaturing the enzyme.

Another proton that can be exchanged under mild conditions is the C-2 proton of histidine. McDonald & Phillips (1970) observed that the resonance due to the C-2 proton of histidine-15 in lysozyme lost intensity if the protein was heated to 65°C in a D_2O solution at neutral pH. The loss in intensity is due to the exchange of the C-2 proton by a deuteron. Meadows et al (1968) used this method to assign the histidine C-2 peaks, observed in the spectrum of ribonuclease-S, to specific histidine residues. This was accomplished by exchanging the C-2 proton of histidine-12 in the S-peptide, and then recombining the S-peptide with the S-protein and obtaining the NMR spectrum of the reconstituted ribonuclease-S.

Although results have been published on the exchange of the C-2 proton in imidazole and substituted imidazoles, no work has been reported for the same exchange in L-histidine. Staub (1964) found that N-benzylimidazole underwent exchange at the 2 position in D_2O at neutral pH. Olafson et al (1964) observed that N,N-dialkylimidazolium salts, that are permanently in the cationic form, underwent rapid exchange at the C-2 carbon in D_2O. Harris & Randall (1965) studied the relationship between rate of protonation

and pH for N-methylimidazole deuterated at the C-2 and C-4 positions. They found the relationship to be in the form of a sigmoidal curve symmetric about the pK of the imidazole, with zero rate at acid pH and a constant rate at alkali pH. The postulated mechanism involved the base catalysed removal of the C-2 deuteron from the conjugate acid of the imidazole, to give a ylide intermediate that obtains a proton from the solvent to give the protonated imidazole. (Results and Discussion Section). Vaughan et al (1970) studied the kinetics of deuteration for imidazole at the C-2 position (at 65°C) and the C-4 position (at 180°C). They obtained similar results to those of Harris & Randall (1965) for exchange at the C-2 position.

In order to obtain information about the kinetics of deuteration at the C-2 position of histidine, a study on L-histidine model compounds was undertaken. The main aim of this work was to see if the rate of deuteration could be varied by changes in the environment around the histidine imidazole ring, with the view to applying the knowledge gained to the deuteration of histidine residues in proteins.

4 B EXPERIMENTAL

The rate of deuteration was measured for imidazole, N-acetyl-L-histidine, L-histidine, β-alanyl-L-histidine and L-histidylglycine. Samples were made up at 5% concentration (w/v) in D_2O. The pH of the solution was measured and adjusted at the reaction temperature by

adding 6 M HCl/D_2O or 6 M NaOD. The solutions were placed into tightly stoppered NMR tubes and the tubes placed in a waterbath set at the required temperature. The pH was measured at the end of the experiment to ensure it had remained constant.

At appropriate intervals the tubes were removed from the waterbath and the NMR spectrum of the sample was obtained on the Perkin-Elmer 60 MHz spectrometer. The proportional area of the C-2 proton peak was determined either by cutting it out on tracing paper and weighing or by measuring the peak height. The weight and height of the peak at zero time was used as a reference equal to one proton. Heights were corrected for changes in instrument resolution by applying the equation

$$H = H2 \times \frac{H4}{H4_o}$$

where H is the corrected height of the C-2 peak, H2 is the measured height of the C-2 peak, H4 is the measured height of the C-4 peak and $H4_o$ is the height of the C-4 peak at zero time. The C-4 proton does not exchange. Both methods, weights and heights, gave results within 5% of each other.

The apparent pK values, used in calculations (Results and Discussion Section), are listed in Table 4.1. The values have been corrected for the deuterium isotope effect by the formula of Glaskoe & Long (1960)

$$pD = pH \text{ (meter reading)} + 0.40$$

TABLE 4.1

	Apparent pK	
	Imidazole group	α-amino group
Imidazole	7.6[a]	
N-acetyl-L-histidine	7.6[a]	
L-histidine	6.6[a]	9.6[a]
β-alanyl-L-histidine	7.4[b]	10.0[b]
L-histidylglycine	6.5[c]	9.0[b]

(a) Sachs et al (1971)

(b) Perrin (1965)

(c) Bradbury & Scheraga (1966)

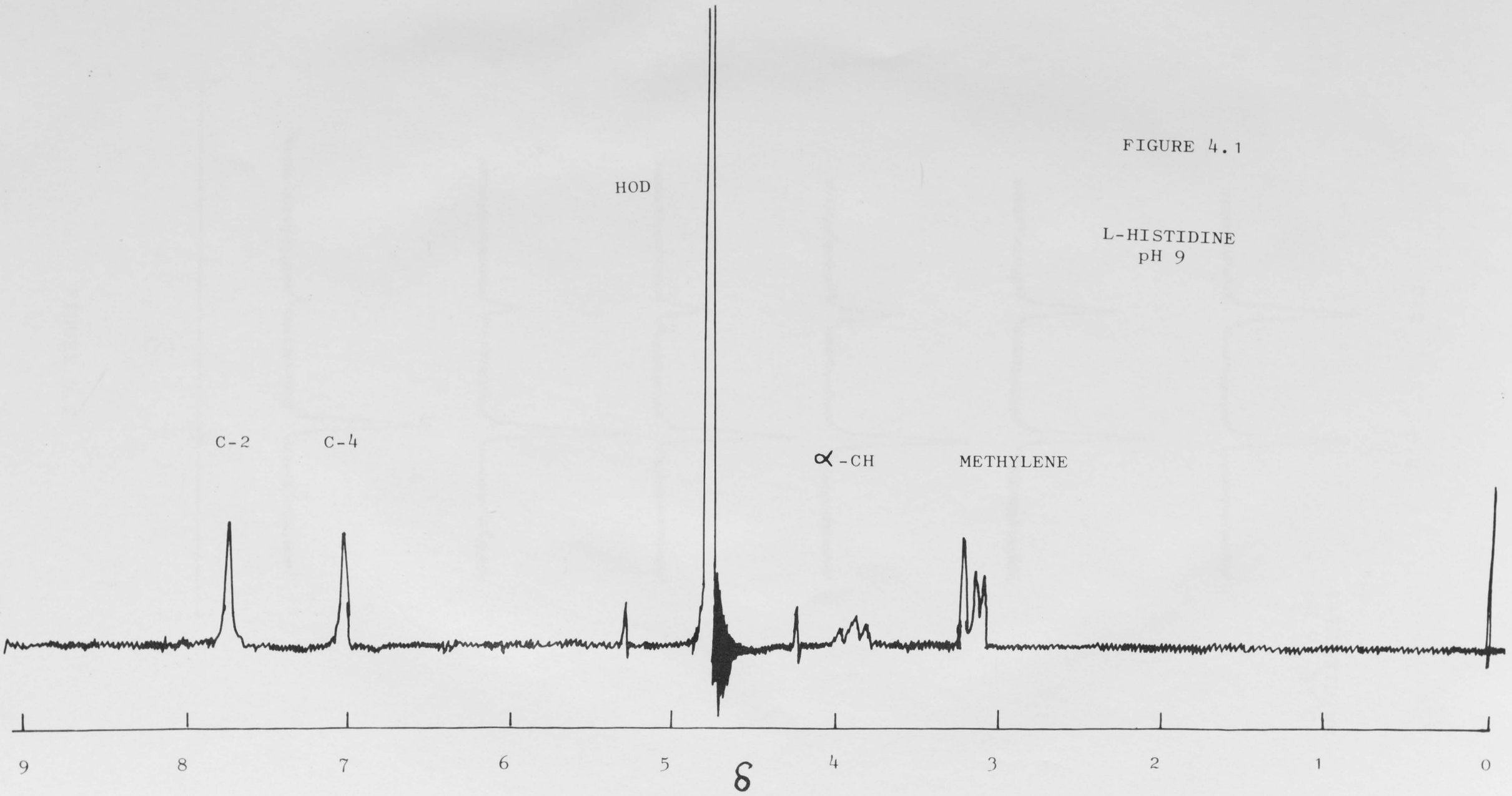
4 C RESULTS AND DISCUSSION

The 60 MHz NMR spectrum of L-histidine in D_2O at pH 9.1 (meter reading) is shown in Figure 4.1 with the peaks assigned to individual protons. A series of spectra obtained during a typical deuteration experiment (pH 9.1, 37°C), of the C-2 and C-4 peaks are shown in Figure 4.2. It can be seen, from the spectra, that the area of the C-2 peak falls with time, while the area of the C-4 peak remains unchanged. The proportional area (A) and its log L(A), for the C-2 peak, is plotted against time in Figure 4.3 (a,b) for a number of pH values at 37°C.

The semi-log plots are linear up to about 80% reaction where an upward deviation is observed. This deviation is caused by back-exchange of C-2 deuterons by protons present in the D_2O solvent. The presence of protons in the solvent arises from a number of sources. A small

FIGURE 4.1

L-HISTIDINE
pH 9



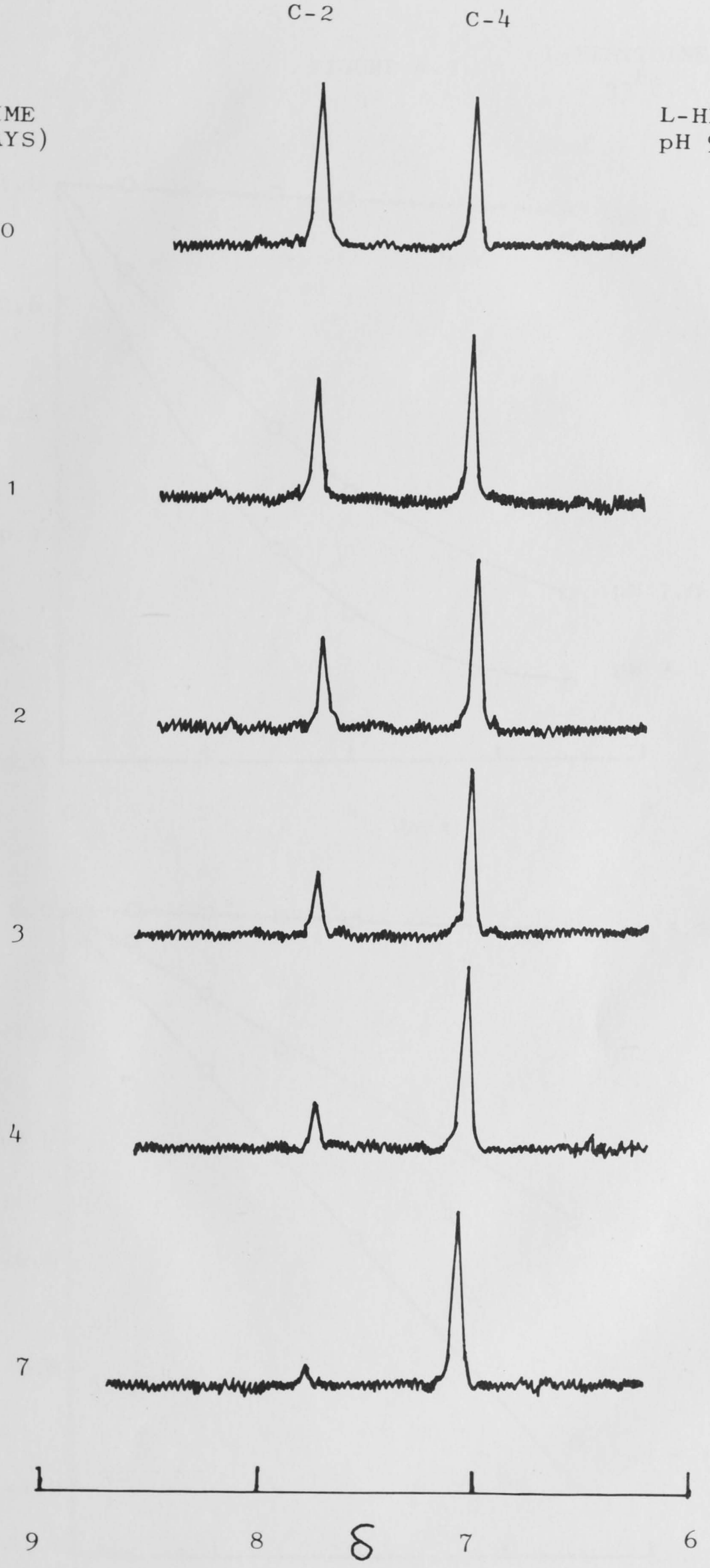
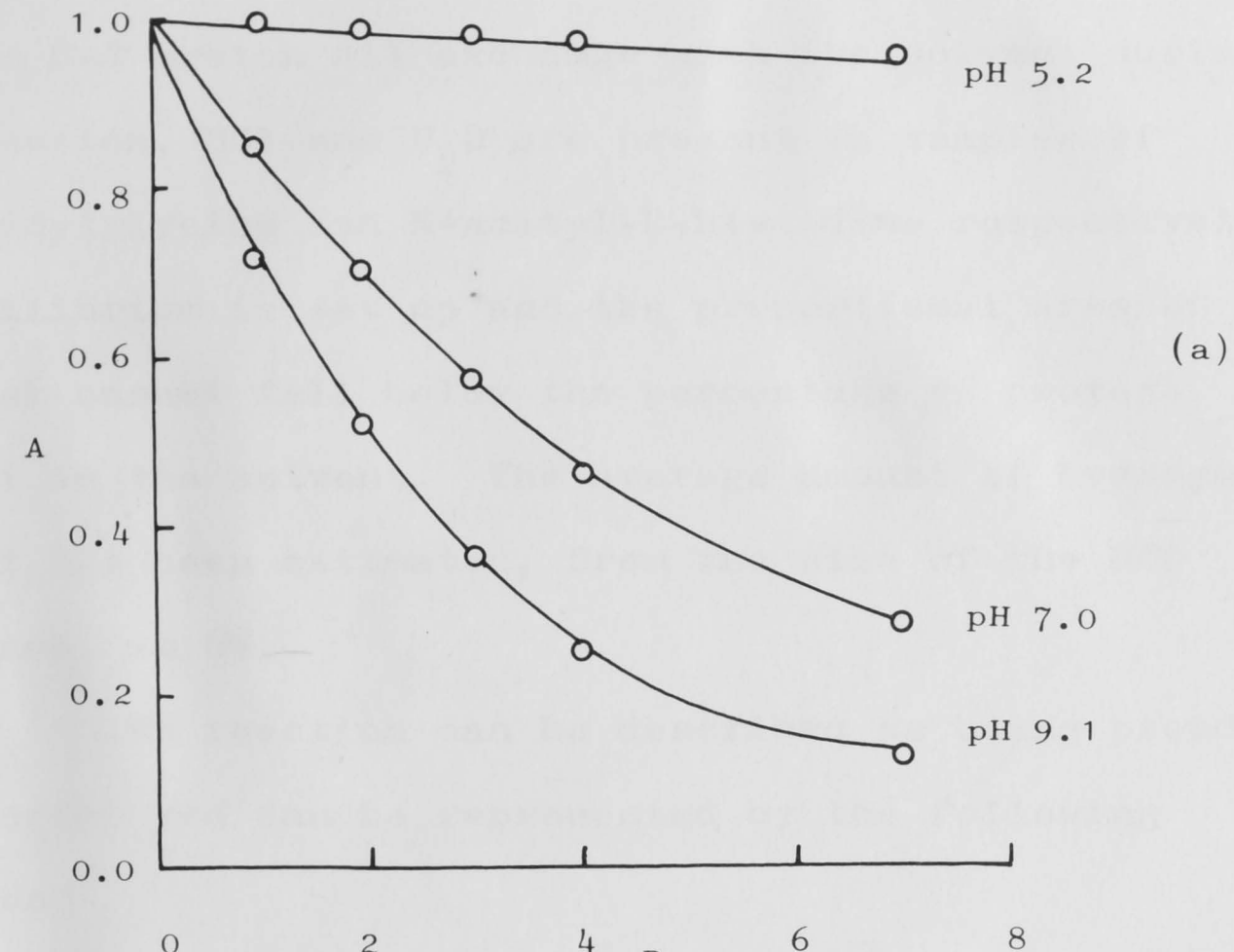
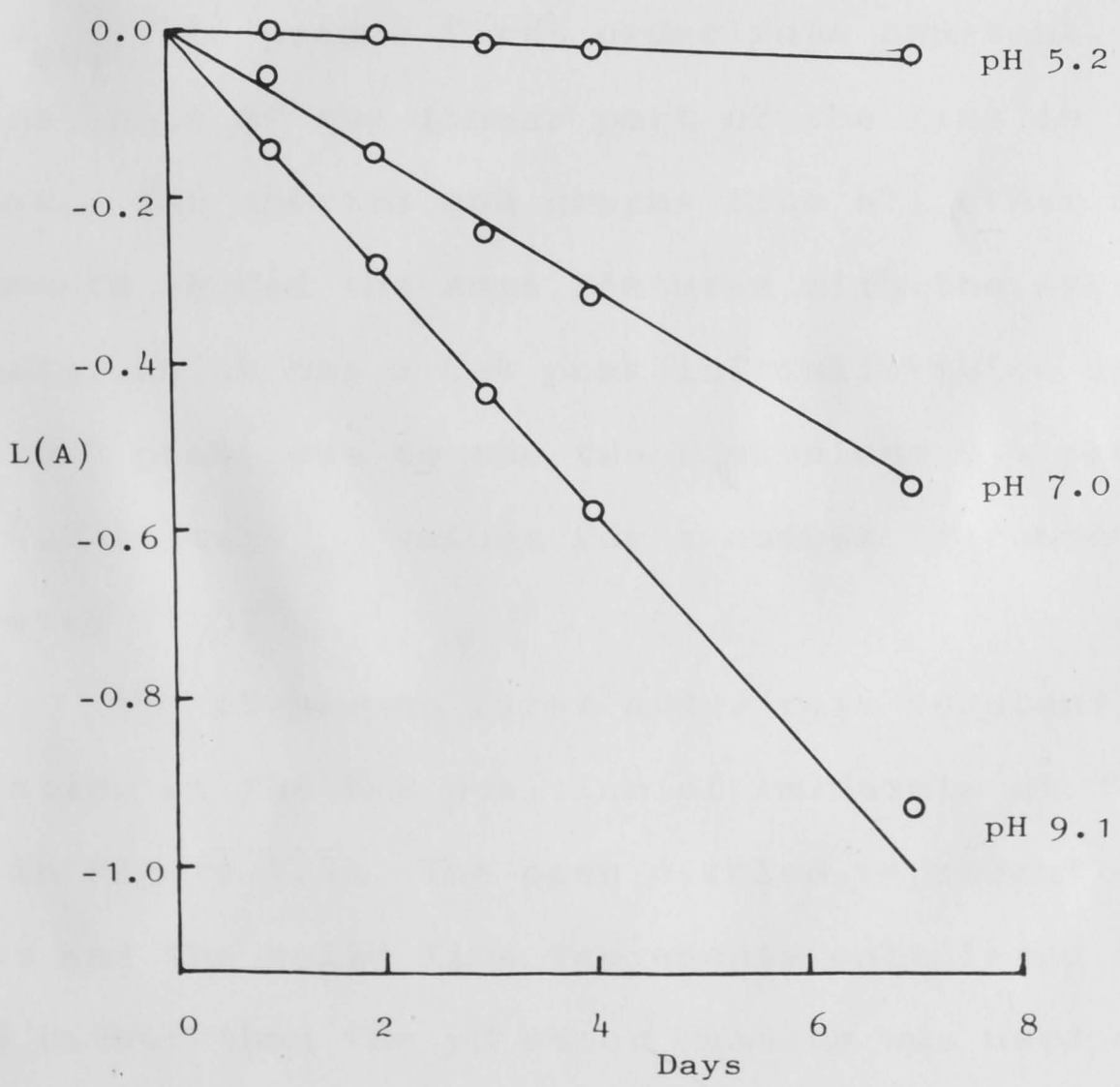


FIGURE 4.2

FIGURE 4.3 L-HISTIDINE
37°C



(a)



(b)

amount is always present in the D_2O solvent and more is introduced during the preparation of the solutions. The α-amino protons, the N-H protons on the imidazole ring and the C-2 proton all exchange with the solvent during the reaction. HCl and H_2O are present in samples of L-histidylglycine and N-acetyl-L-histidine respectively. An equilibrium is set up and the proportional area of the C-2 peak cannot fall below the percentage of protons present in the solvent. The average amount of hydrogen present has been estimated, from the size of the HOD resonance, at 6%.

The reaction can be described as being pseudo first order and can be represented by the following equation

$$\text{Rate} = k_{obs} \left[\text{Histidine}\right]$$

where k_{obs}, the pseudo first order rate constant, is obtained from the slope of the linear part of the line in the semi-log plot. The spectra and graphs from all other deuteration experiments showed the same features with the exception of imidazole, which has a C-4 peak initially twice as large as the C-2 peak, due to the two equivalent C-4 protons. Table 4.2 lists k_{obs} values for a number of compounds deuterated at 37°C.

The pD-pseudo first order rate constant plot, for deuteration at the C-2 position of imidazole at 37°C, is shown in Figure 4.4a. The open circles represent experimental results and the solid line represents calculated results. The pD rather than the pH meter reading was used as the

TABLE 4.2

The units of k_{obs} are $days^{-1}$. The reaction temperature is 37°C.

Imidazole		N-acetyl-L-histidine		L-histidylglycine	
pD	k_{obs}	pD	k_{obs}	pD	k_{obs}
3.1	0.00	3.9	0.00	5.4	0.01
7.0	0.28	6.6	0.13	7.4	0.19
8.8	1.03	8.2	0.38	8.5	0.26
10.4	1.20	8.9	0.56	9.6	0.49
12.5	1.23	10.4	0.63	11.4	0.66
13.4	1.20	12.9	0.65		

β-alanyl-L-histidine

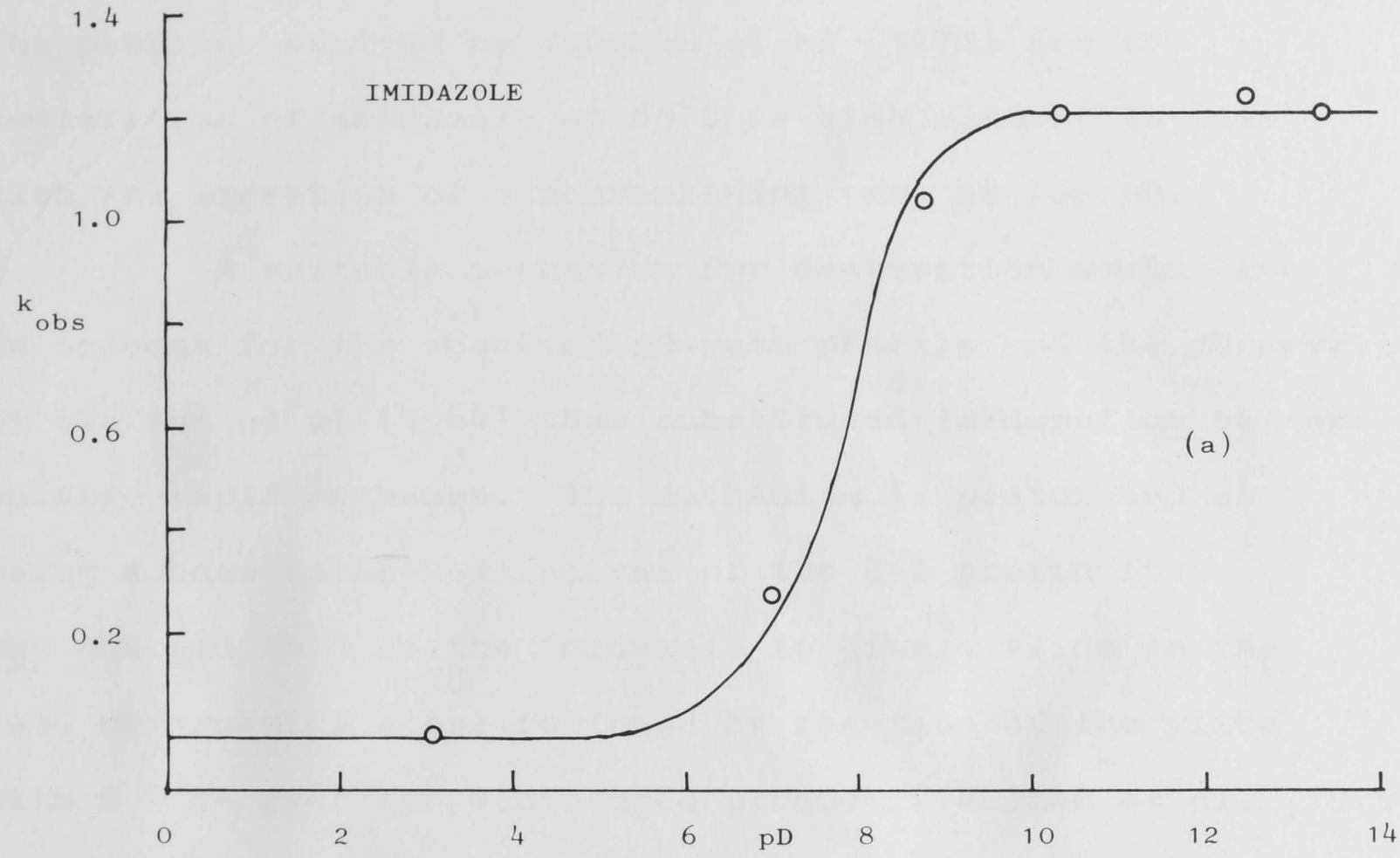
pD	k_{obs}
5.2	0.00
6.4	0.08
7.6	0.35
8.6	0.47
9.0	0.47
9.3	0.51
9.9	0.57
10.4	0.63
12.2	0.65
13.4	0.65

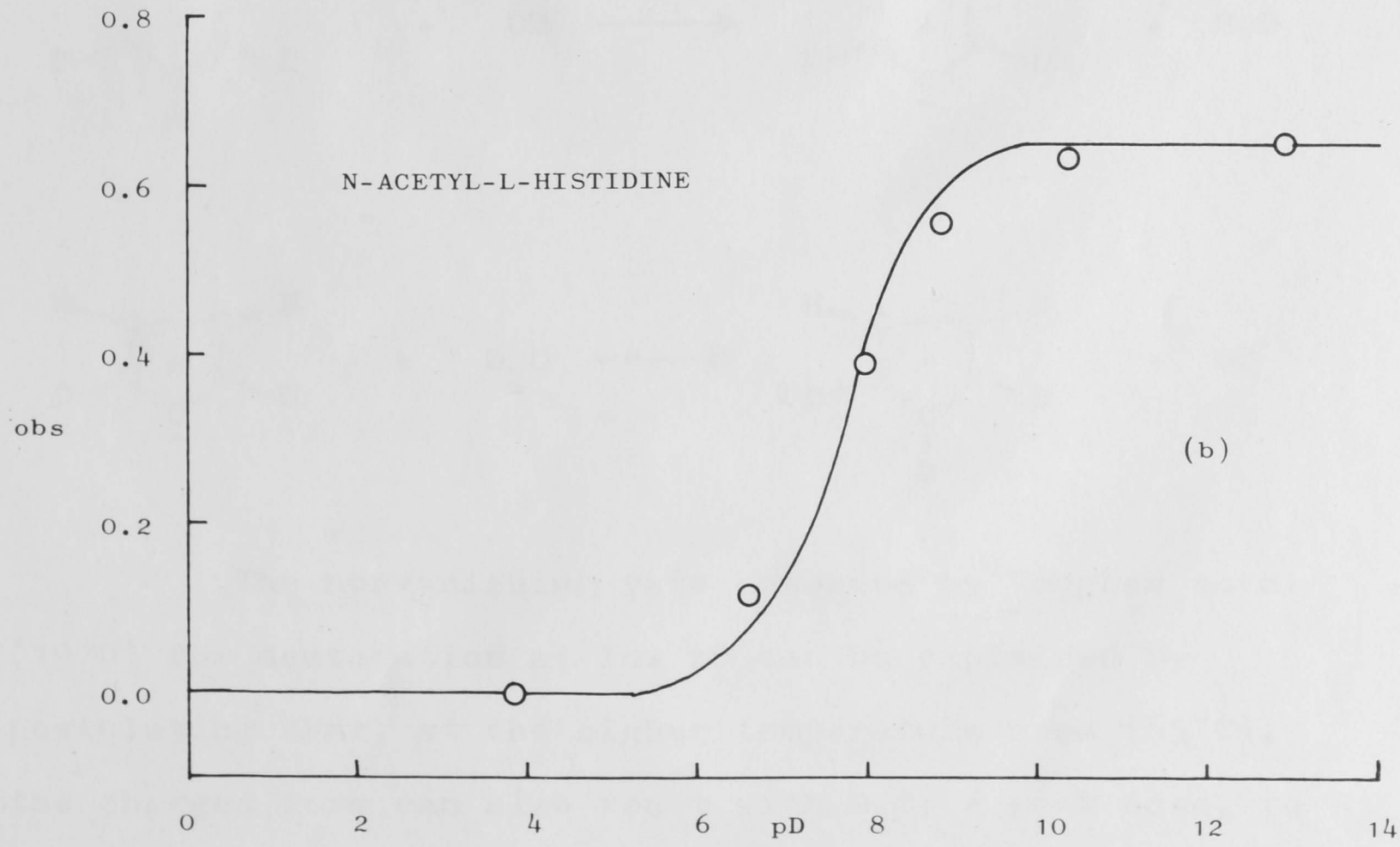
TABLE 4.2 (cont.)

L-histidine

pD	k_{obs}
1.2	0.01
5.5	0.03
6.8	0.14
7.2	0.16
7.4	0.25
7.9	0.29
8.2	0.27
8.6	0.29
9.4	0.32
9.5	0.40
9.9	0.56
10.9	0.71
11.4	0.65
12.4	0.69
12.9	0.70
13.4	0.69

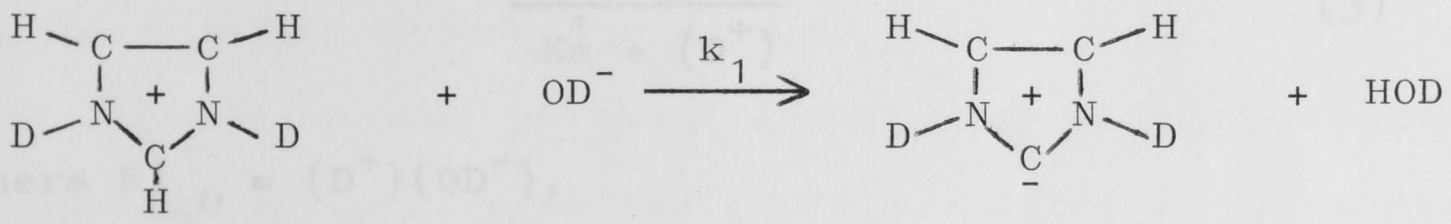
FIGURE 4.4

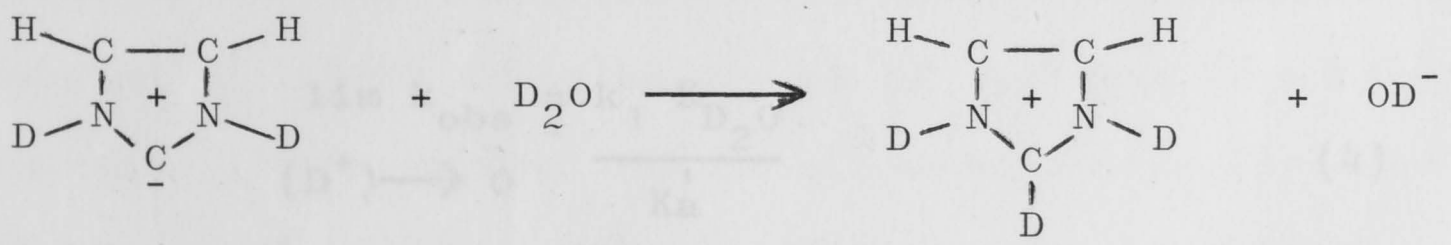

1.4
IMIDAZOLE
1.0
k_{obs}
0.6
(a)
0.2
0
2
4
6
pD
8
10
12
14


0.8
0.6
N-ACETYL-L-HISTIDINE
0.4
obs
(b)
0.2
0.0
0
2
4
6
pD
8
10
12
14

latter is not a measure of the deuterium ion concentration. This pD-rate profile is the same as that observed by Harris & Randall (1965) for the protonation of N-methylimidazole-3d. The profile observed by Vaughan et al (1970) for the deuteration of imidazole at 65°C is also similar to this with the exception of a nonvanishing rate at low pD.

A suitable mechanism for deuteration would have to account for the observed pD-rate profile and the observation by Olafson et al (1964) that substituted imidazolium cations undergo rapid exchange. The mechanism is postulated as being a base catalysed removal of the C-2 proton from the charged form of the imidazole to give a ylide in the rate determining step, followed by reaction of the ylide with D_2O to give the deuterated product (Vaughan et al, 1970).





The nonvanishing rate observed by Vaughan et al (1970) for deuteration at low pD can be explained by postulating that, at the higher temperature used (65°C), the charged form can also react with D_2O, a weak base, to give the ylide.

A discussion of alternate deuteration mechanisms is given in Appendix IIC.

This mechanism leads to the equation:-

$$\text{Rate} = k_1 (OD^-)(Imid^+) = k_{obs}(Imid^t) \qquad (1)$$

where k_1 is the second order rate constant, $Imid^+$ is the concentration of the charged form and $Imid^t$ is the total concentration of imidazole.

$$\text{As} \quad Imid^+ = \frac{(Imid^t)(D^+)}{K\!a' + (D^+)} \qquad (2)$$

where $K\!a'$, the apparent dissociation constant, is given by the equation:-

$$K\!a' = \frac{(Imid)(D^+)}{(Imid^+)}$$

where Imid is the concentration of the uncharged form of imidazole, equation (1) can be rearranged to give

$$k_{obs} = \frac{k_1 \; K_{D_2O}}{K\!a' + (D^+)} \qquad (3)$$

where $K_{D_2O} = (D^+)(OD^-)$.

At high pD, $(D^+) \ll K\!a'$, so that

$$\lim_{(D^+) \longrightarrow 0} k_{obs} = \frac{k_1 \; K_{D_2O}}{K\!a'} \qquad (4)$$

The value of the second order rate constant k_1, can be calculated by using the experimental values of k_{obs} obtained at high pD. Equation (3) can then be used to derive the calculated curve. The value of K_{D_2O} at 37°C is taken as being 3.30×10^{-15} (Handbook of Physics and Chemistry, 1968). The value of $K\!a'$ is obtained from the apparent

pK in Table 4.1. Values of the second order rate constant, for the deuteration of a number of compounds at 37°C, are given in Table 4.3.

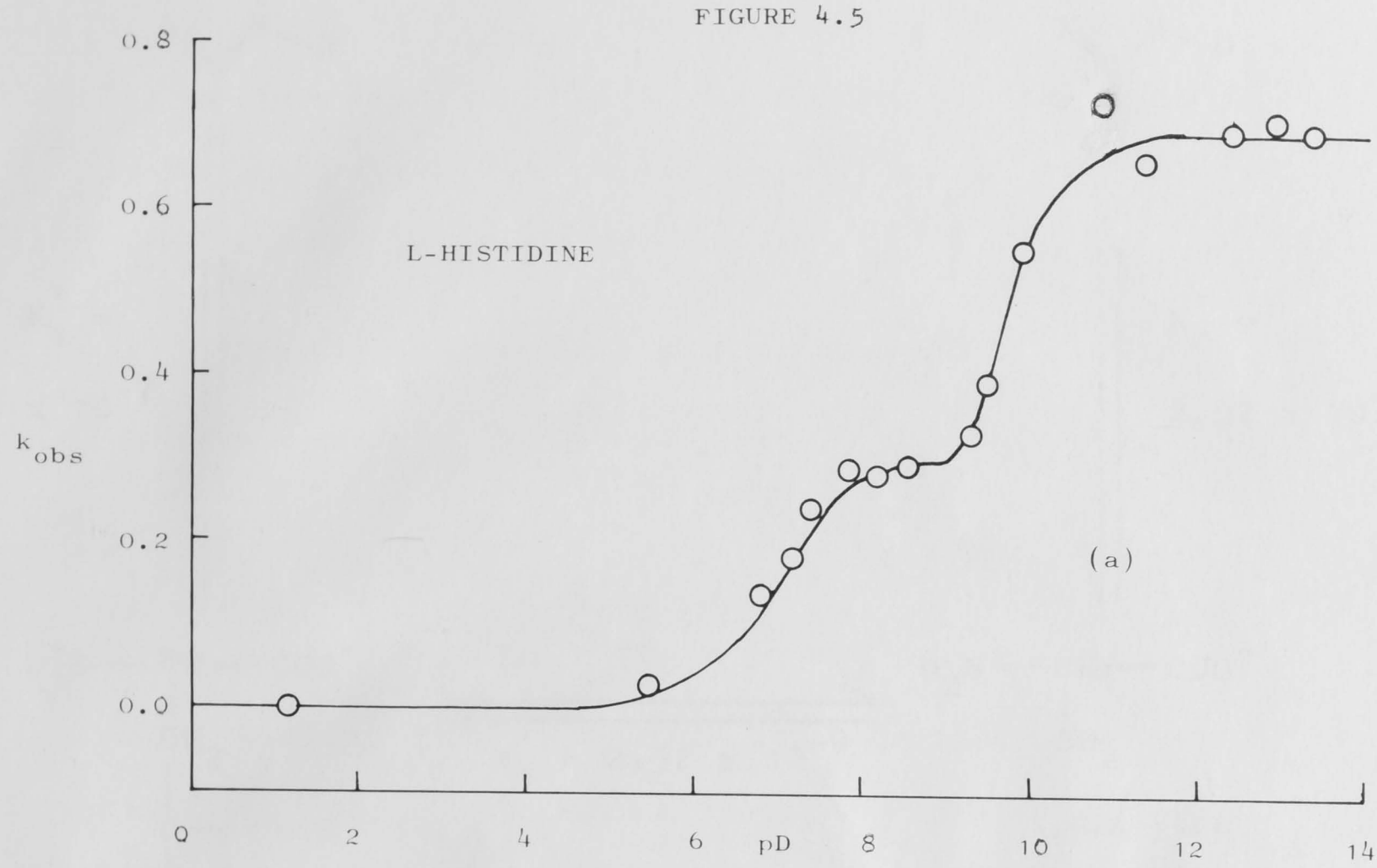
The experimental results, open circles, and the calculated results, solid line, for the deuteration of N-acetyl-L-histidine are shown in Figure 4.4b. The experiments were carried out at 37°C. The pD-rate profile has the same shape as that observed for imidazole.

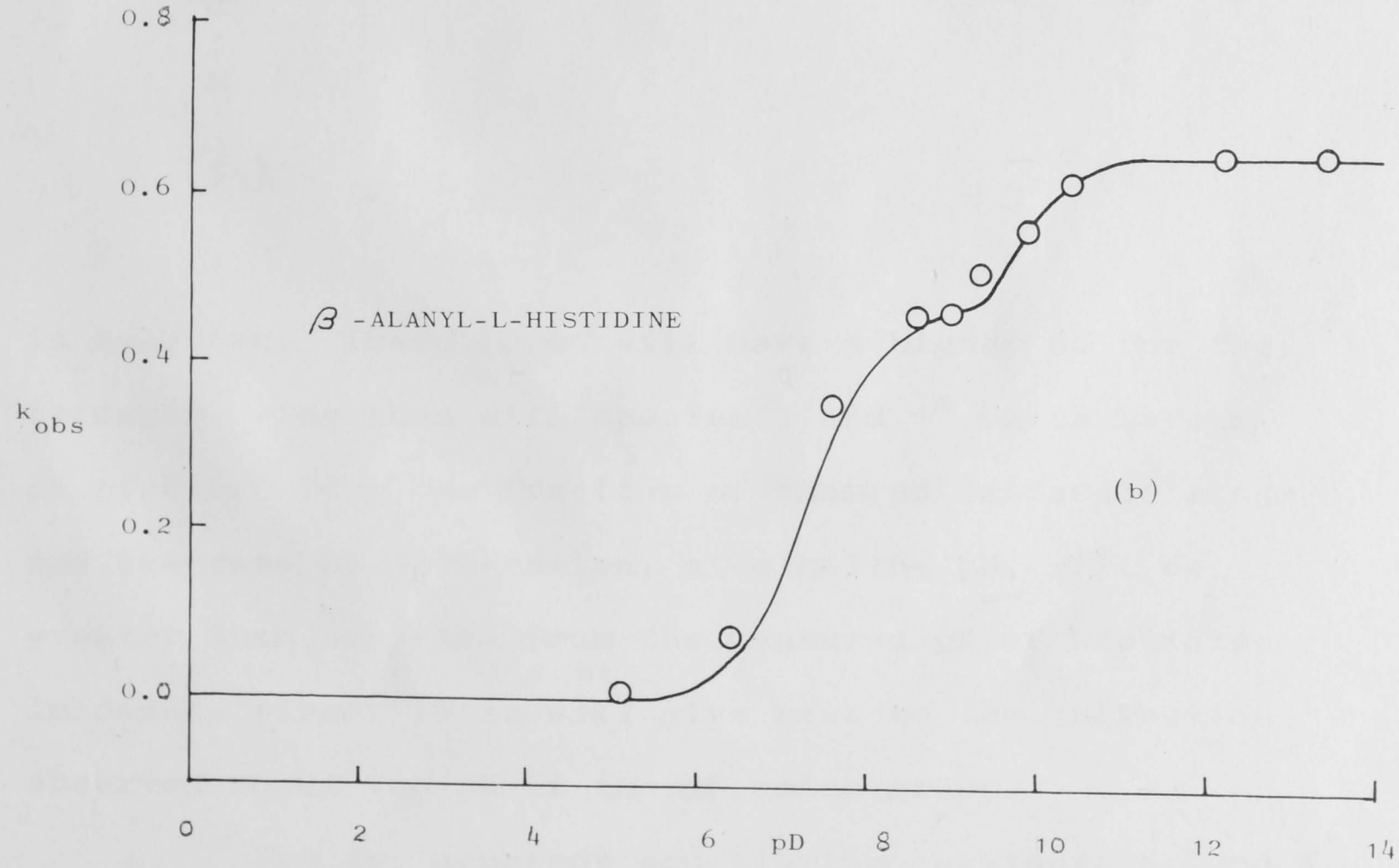
The results for the deuteration at 37°C of L-histidine and β-alanyl-L-histidine are shown in Figure 4.5 (a,b). The pD-rate profile differs from that observed for imidazole and N-acetyl-L-histidine, as a second inflection is present at pD 9.6 and 10.0 respectively. This result can be explained by considering the various species of L-histidine present at neutral and alkaline pD as shown in the following diagram.

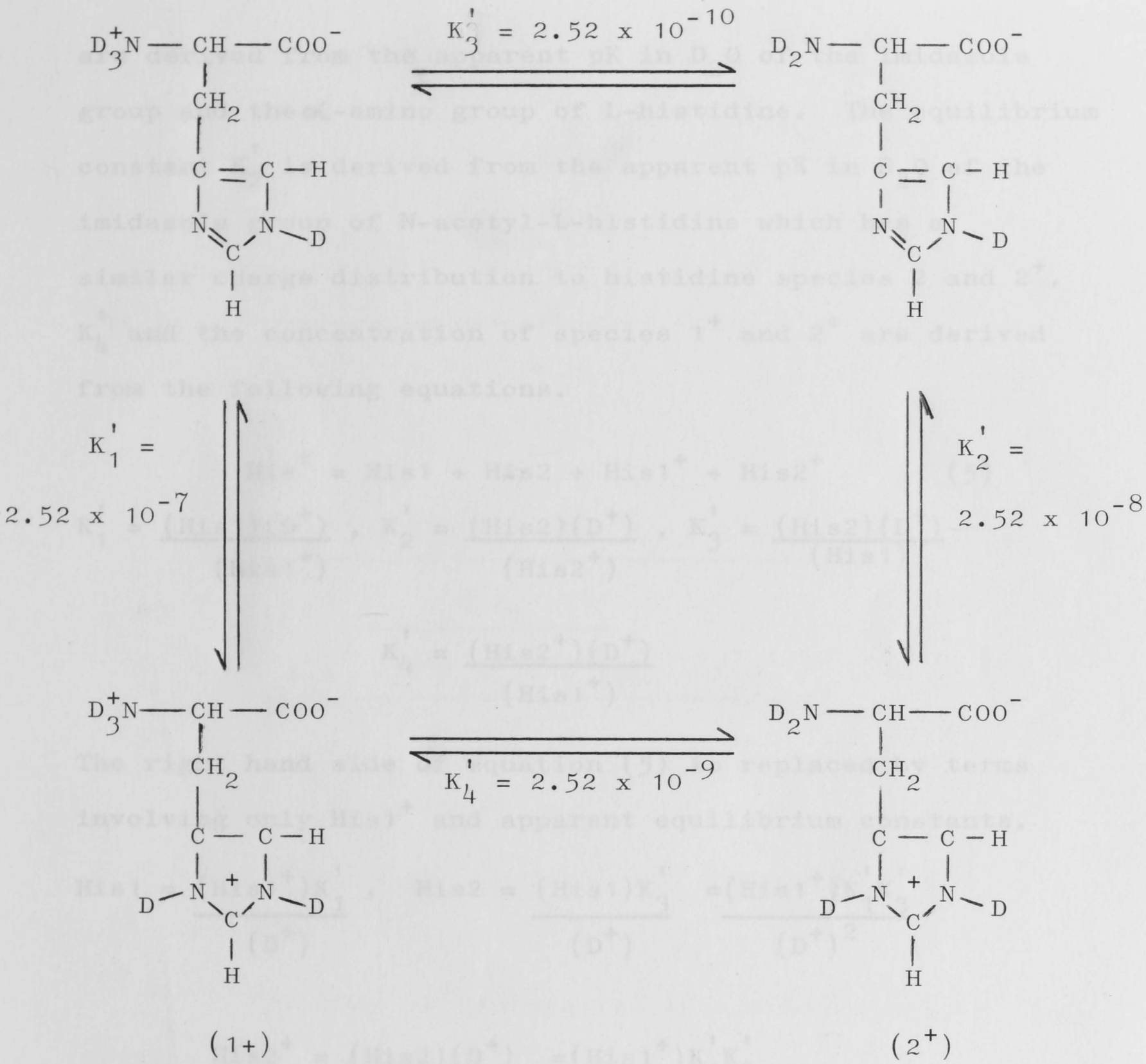
At neutral pD the α-amino group of L-histidine is charged while at alkaline pD (i.e. pD 11) it is uncharged. The positive charge on the α-amino group has a repulsive effect on the N-D deuterons of the charged imidazole ring (species 1^+), thus lowering the pK of the ring (Bradbury & Scheraga, 1966). Removal of the α-amino charge (species 2^+) raises the pK of the imidazole ring, as can be seen for the model compound N-acetyl-L-histidine where the pK increases from 6.6 (for histidine) to 7.6.

At any given pD the fraction of charged imidazole rings is dependent on their pK, and will increase with increasing pK. Above pD 11, species 2 and 2^+ predominate

FIGURE 4.5


0.8
0.6
0.4
0.2
0.0
k_{obs}
L-HISTIDINE
(a)
0
2
4
6
pD
8
10
12
14


0.8
0.6
0.4
0.2
0.0
k_{obs}
β -ALANYL-L-HISTIDINE
(b)
0
2
4
6
pD
8
10
12
14



in solution. These forms will have a higher pK for the imidazole ring than will species 1 and 1^+ which have a pK of 6.6. Thus the fraction of charged imidazole rings and the rate of deuteration, at alkaline pD, will be greater than expected from the measured pK of histidine imidazole rings. This will give rise to the inflection observed about the pK of the α-amino group.

The two apparent equilibrium constants K_1' and K_3'

are derived from the apparent pK in D_2O of the imidazole group and the α-amino group of L-histidine. The equilibrium constant K_2' is derived from the apparent pK in D_2O of the imidazole group of N-acetyl-L-histidine which has a similar charge distribution to histidine species 2 and 2^+. K_4' and the concentration of species 1^+ and 2^+ are derived from the following equations.

$$His^t = His1 + His2 + His1^+ + His2^+ \qquad (5)$$

$$K_1' = \frac{(His1)(D^+)}{(His1^+)} \quad , \quad K_2' = \frac{(His2)(D^+)}{(His2^+)} \quad , \quad K_3' = \frac{(His2)(D^+)}{(His1)}$$

$$K_4' = \frac{(His2^+)(D^+)}{(His1^+)}$$

The right hand side of equation (5) is replaced by terms involving only $His1^+$ and apparent equilibrium constants.

$$His1 = \frac{(His1^+)K_1'}{(D^+)} \quad , \quad His2 = \frac{(His1)K_3'}{(D^+)} \quad = \frac{(His1^+)K_1'K_3'}{(D^+)^2}$$

$$His2^+ = \frac{(His2)(D^+)}{K_2'} \quad = \frac{(His1^+)K_1'K_3'}{(D^+)K_2'}$$

Thus:-

$$His^t = His1^+\left[\frac{K_1'}{D^+} + \frac{K_1'K_3'}{D^{+\,2}} + 1 + \frac{K_1'K_3'}{K_2'D^+}\right] \qquad (6)$$

The right hand side of equation (1) may be solved for $His2^+$ and the apparent equilibrium constants in a similar manner.

$$His^t = His2^+ \left[\frac{K_2'}{K_3'} + \frac{K_2'}{D^+} + \frac{K_2'D^+}{K_1'K_3'} + 1 \right] \qquad (7)$$

$$K_4' = \frac{D^+ \left[\frac{K_1'}{D^+} + \frac{K_1'K_3'}{D^{+\,2}} + 1 + \frac{K_1'K_3'}{K_2'D^+} \right]}{\frac{K_2'}{K3'} + \frac{K_2'}{D^+} + \frac{K_2'D^+}{K_1'K_3'} + 1}$$

$$= \frac{K_1'K_2'D^+ + K_1'K_2'K_3' + D^{+\,2}K_2' + D^+K_1'K_3'}{\frac{D^+K_2'}{K_1'K_2'K_3' + D^+K_1'K_2' + D^+K_1'K_3' + K_2'D^{+\,2}}{D^+K_1'K_3'}}$$

$$= \frac{K_1'K_3'}{K_2'}$$

The rate of deuteration of histidine may be written as:-

$$\text{Rate} = k_{obs}(His^t) = k_1(OD^-)(His1^+) + k_2(OD^-)(His2^+).$$

Rearranging this equation gives:-

$$k_{obs} = \frac{K_{D_2O}\left[k_1(His1^+) + k_2(His2^+)\right]}{(D^+)(His^t)} \qquad (8)$$

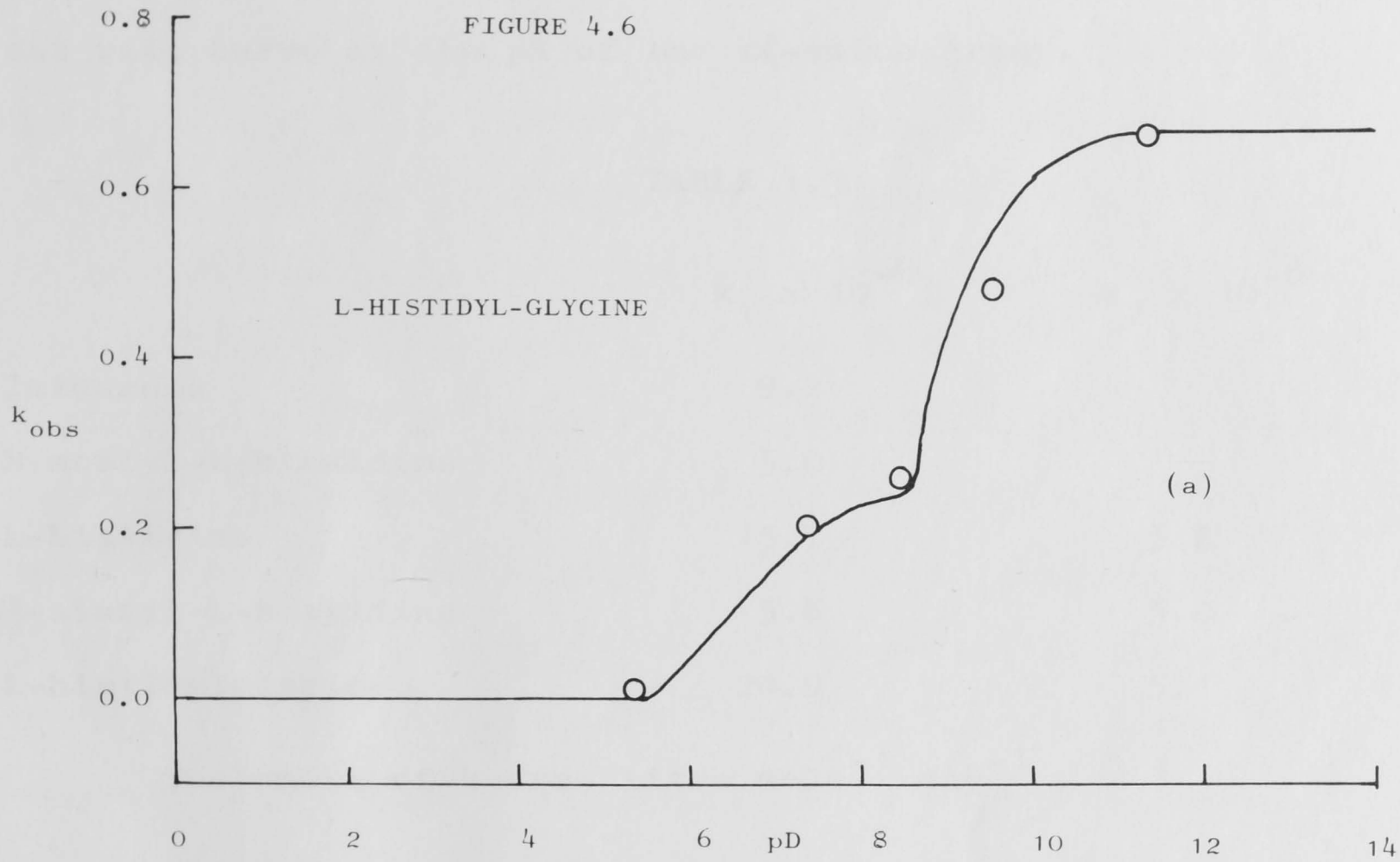
The concentrations of species 1^+ and 2^+ are calculated from equations (6) and (7). The second order rate constants k_1 and k_2 are evaluated from equation (4). The limiting value of k_{obs}, at alkali pD, is used to determine the

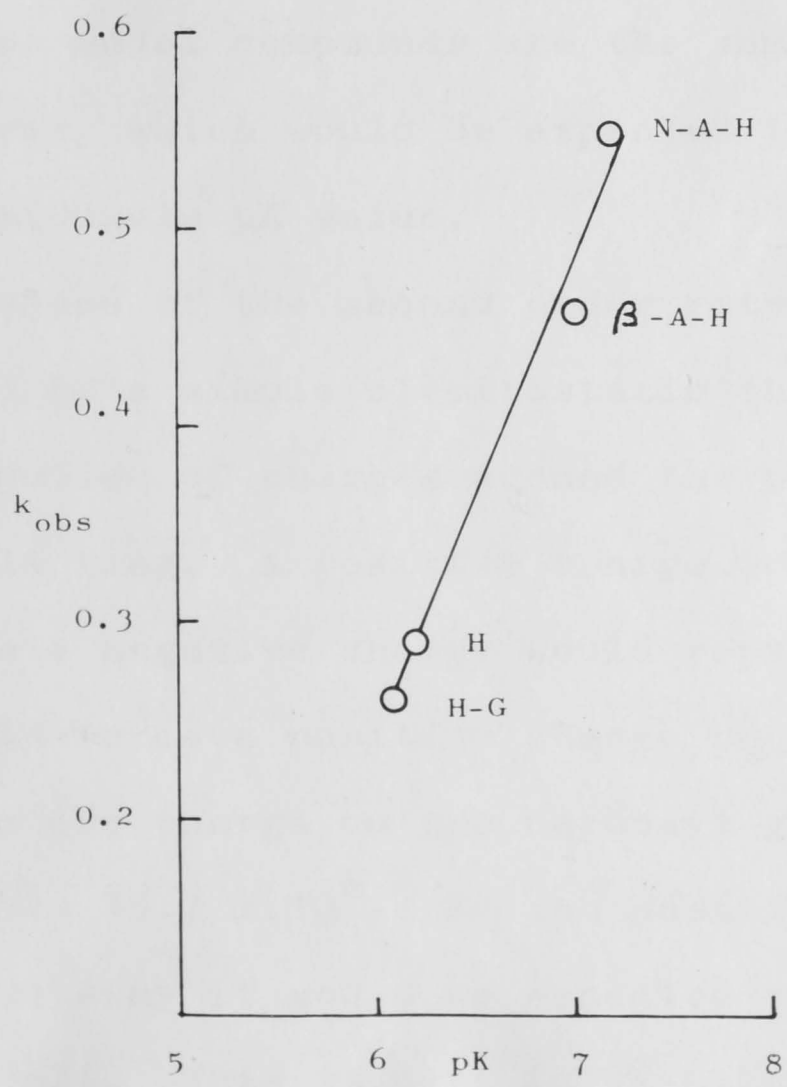
value of k_2. The value of k_1 is more difficult to determine. A number of limiting k_{obs} values are chosen and the corresponding k_1 values are determined using equation (4). The limiting k_{obs} values may be regarded as those that would be reached if the α-amino group did not lose its charge at high pD. A number of calculated lines are derived using equation (8). The correct value of k_1 is that which gives the best fit of calculated line to experimental results. Values of k_1 and k_2 are given in Table 4.3. There is a good fit between the calculated line and the experimental results indicating that the postulated mechanism for the reaction is correct.

The results for β-alanyl-L-histidine were treated in the same manner as for L-histidine, using values of $K_1' = 4.0 \times 10^{-8}$, $K_3' = 1 \times 10^{-10}$ (obtained from the apparent pK values in Table 4.1) and $K_2' = 2.52 \times 10^{-8}$ (obtained from the apparent pK of N-acetyl-L-histidine). K_4' is equal to 1.60×10^{-10}. Values of k_1 and k_2 are given in Table 4.3. As the charged α-amino group on the β-alanyl residue is further away from the imidazole ring than in L-histidine, its removal does not have as large an effect on the pK of the imidazole ring and the rate of deuteration as in L-histidine.

Results for L-histidylglycine are presented in Figure 4.6 (a). The calculated line may not be very accurate due to the small number of experimental points. Values for equilibrium constants are $K_1' = 3.16 \times 10^{-7}$, $K_3' = 1 \times 10^{-9}$ (obtained from the apparent pK values in Table 4.1) and $K_2' = 2.52 \times 10^{-8}$ (obtained from the apparent

FIGURE 4.6


0.8
0.6
0.4
0.2
0.0
k_{obs}
L-HISTIDYL-GLYCINE
(a)
0
2
4
6
pD
8
10
12
14


0.6
0.5
0.4
0.3
0.2
k_{obs}
N-A-H
β-A-H
H
H-G
5
6
pK
7
8

(b)

pK of N-acetyl-L-histidine). The value of K_4' is 1.25×10^{-8}. As the charged α-amino group is close to the imidazole ring, its removal results in a prominent inflection in the rate curve at the pK of the α-amino group.

TABLE 4.3

	$k_1 \times 10^{-6}$	$k_2 \times 10^{-6}$
Imidazole	9.2	
N-acetyl-L-histidine	5.0	
L-histidine	15.7	5.2
β-alanyl-L-histidine	5.8	5.0
L-histidylglycine	20.0	5.1

The units of k are litre $mole^{-1}$ day^{-1}

The results for the limiting value of k_{obs} for all the histidine model compounds are the same, within experimental error, which would be expected if they all have the same imidazole pK value.

The values of the second order rate constants can be explained by a simple electrostatic theory dealing with the distribution of charges around the positively charged imidazole ring. A positive charge would attract an OD^- ion while a negative charge would repel the ion. In L-histidine there is a positive charge on the α-amino group and a negative charge on the carboxyl group, and the value of k_1 is 15.7×10^6. If the positive charge is moved one residue away it would be expected that the rate constant would fall. This is seen in β-alanyl-L-histidine

where k_1 is 5.8×10^6. Complete removal of this positive charge would be expected to lower the rate constant even further. This is seen in N-acetyl-L-histidine, where k_1 is 5.0×10^6, and in L-histidine and β-alanyl-L-histidine where k_2 is 5.2 and 5.0×10^6. If the negative charge is moved one residue away, it would be expected that the rate constant would be larger than for L-histidine. This is seen in L-histidylglycine where k_1 is 20.0×10^6. However, it would also be expected that k_2 for L-histidylglycine would be larger than that for the other histidine compounds. As this value was calculated from a single experimental point, it may be in error. Complete removal of the negative charge would result in an increase in the rate constant over that observed for histidine compounds with a negative charge on the carboxyl group. This is seen in imidazole where the rate constant increases from about 5.1 to 9.2×10^6.

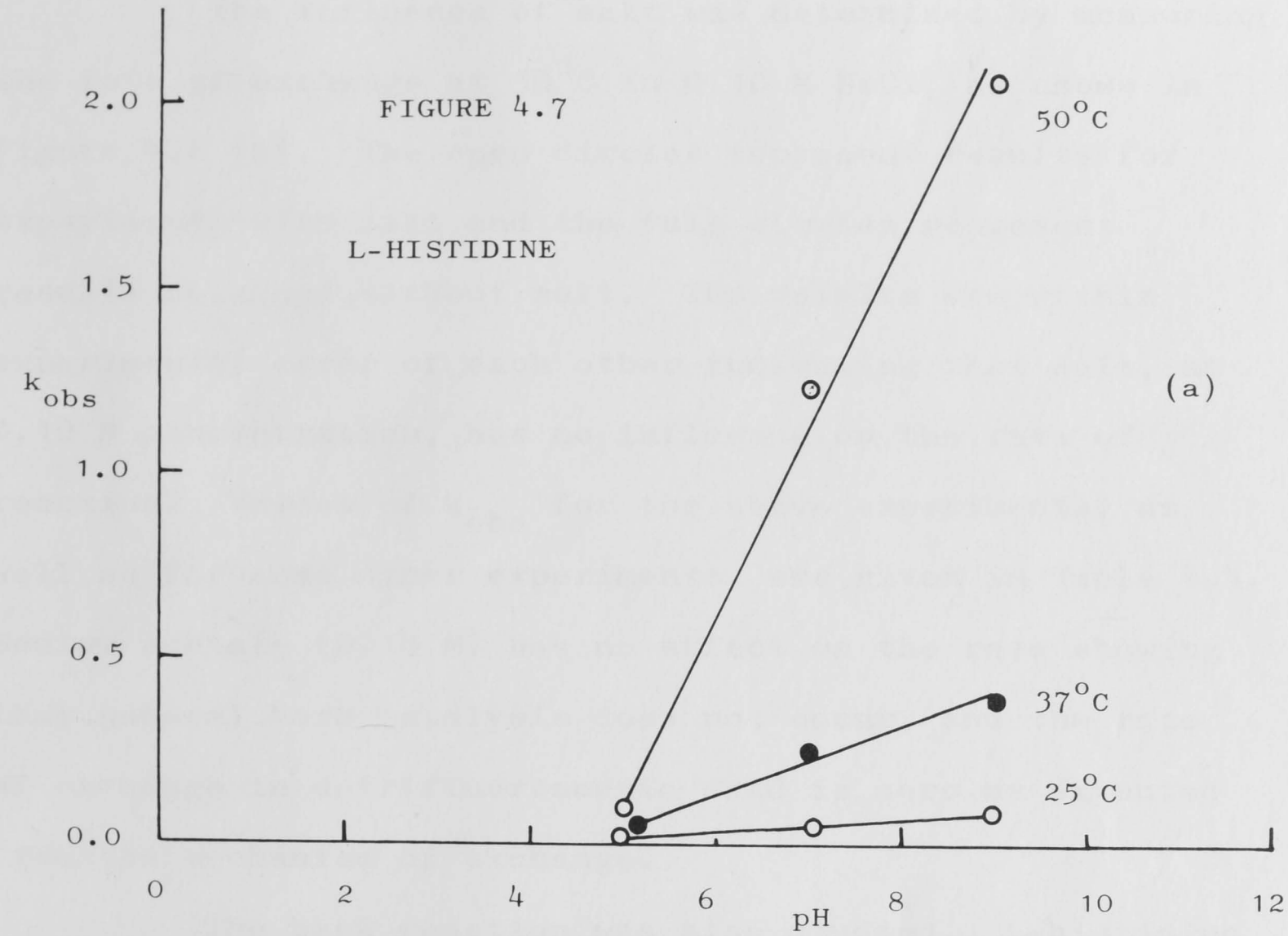
The overall rate of exchange is dependent on both the pK of the imidazole ring and the second order rate constant. In L-histidine, although k_2 is only one third as large as k_1, the increase in pK of one unit results in a ten-fold increase in the fraction of charged imidazole rings at high pD which gives an increase in the overall rate of exchange. Figure 4.6 (b) is a plot of k_{obs} vs pK, uncorrected for deuterium isotope effects, for N-acetyl-L-histidine (N-A-H), β-alanyl-L-histidine (β-A-H), L-histidine (H) and L-histidylglycine (H-G), at pH meter reading 8.1-8.5. The rate of exchange increases with pK. It may be possible to differentially exchange

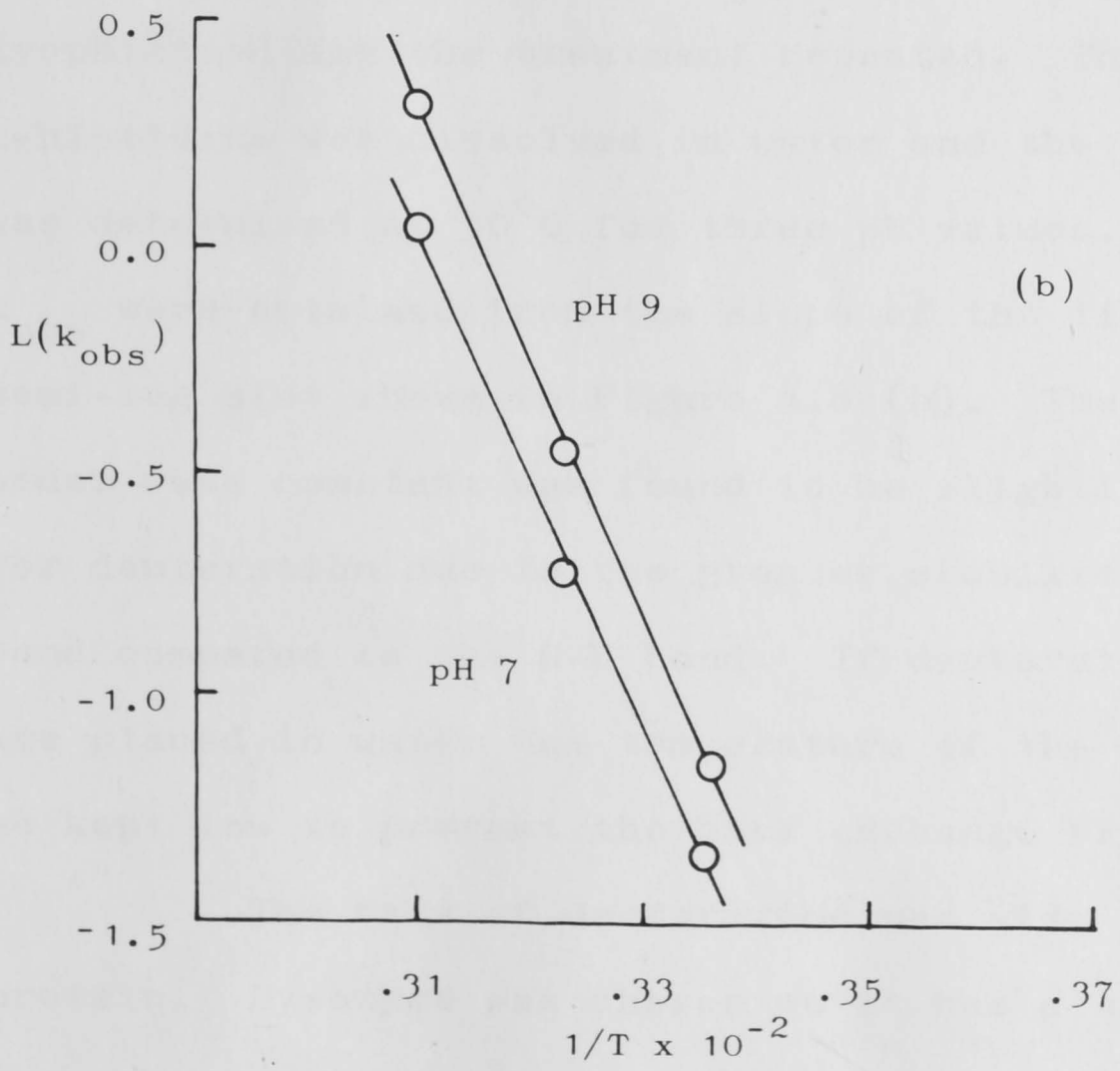
A discussion of the differential deuteration of histidine derivatives is given in Appendix IID.

C-2 protons of histidine residues in proteins provided the pK's are sufficiently different and the second order rate constants are approximately the same. It could then be possible, if a large enough difference is observed, to assign the C-2 resonances to the different histidine residues in a protein by splitting the protein and obtaining the NMR spectra of the peptides.

Experiments were carried out to investigate the effect of temperature, salts and d-trifluoroacetic acid on the rate of exchange in L-histidine. The relationship between k_{obs} and temperature is shown in Figure 4.7 (a). There is a marked increase in rate with increasing temperature. Straight lines have been drawn through the points as there are insufficient results to draw the sigmoidal curve. The Arrhenius activation energy, obtained from the slope of the line log k_{obs} vs $1/T$ where T is the absolute temperature, is 24.5 ± 4 Kcal at pH meter reading 7, and 25.0 ± 3 Kcal at pH meter reading 9, giving an average value of 24.8 ± 4 Kcal. Vaughan et al (1970) give a value for imidazole, determined over a 10 C^{o} range, of 11 ± 3 Kcal. The activation energy for the deuteration of imidazole is lower than in L-histidine. The greater rate of reaction observed for imidazole, Table 4.2, could be due to the lower energy of activation for the deuteration reaction. If this is the reason for the greater reaction rate, then the results for imidazole cannot be used in the electrostatic theory developed to explain the second order rate constants in Table 4.3.

FIGURE 4.7


L-HISTIDINE
2.0
1.5
1.0
0.5
0.0
k_{obs}
50°C
37°C
25°C
(a)
0
2
4
6
8
10
12
pH

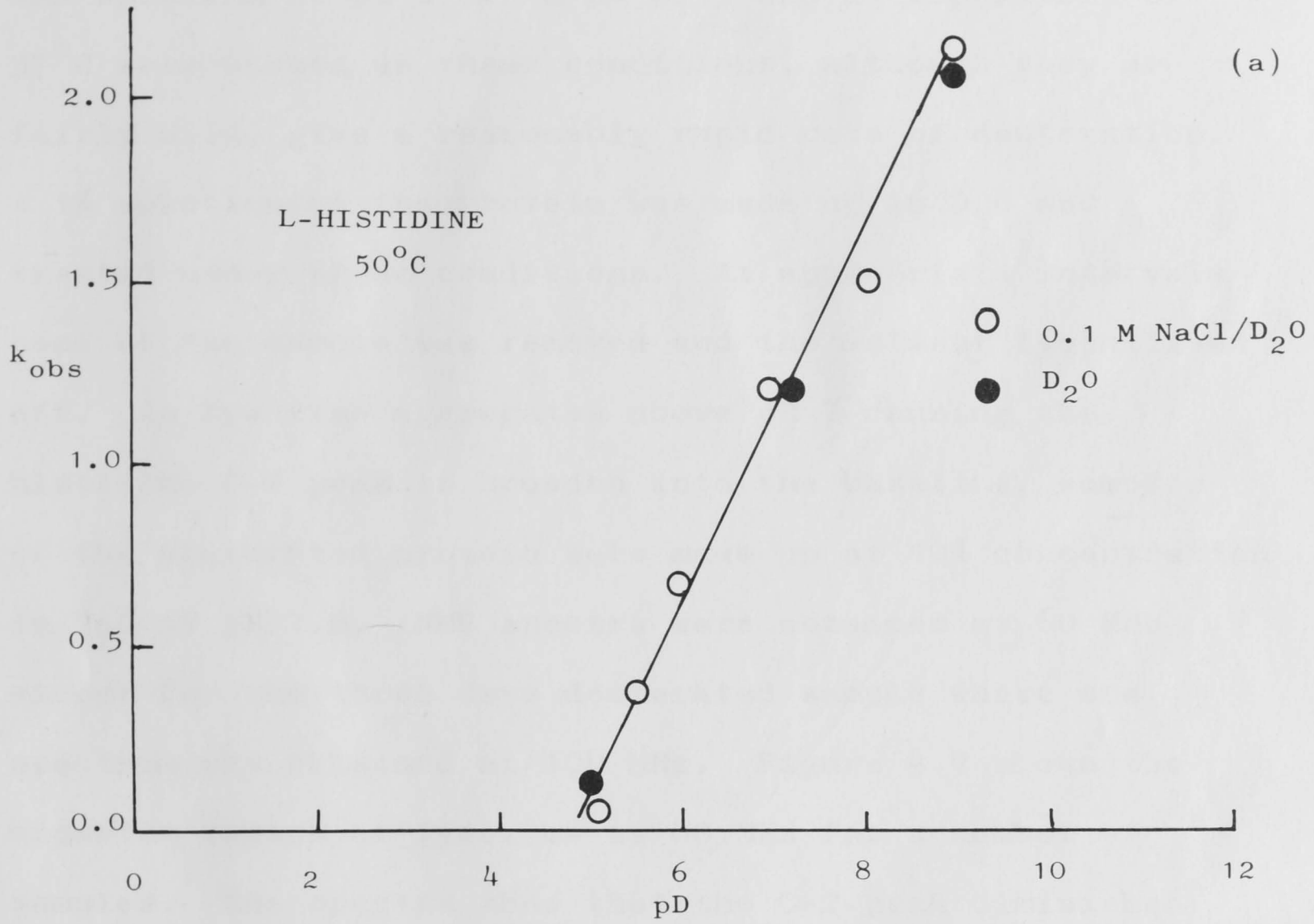

0.5
0.0
-0.5
-1.0
-1.5
$L(k_{obs})$
pH 9
pH 7
(b)
.31
.33
.35
.37
$1/T \times 10^{-2}$

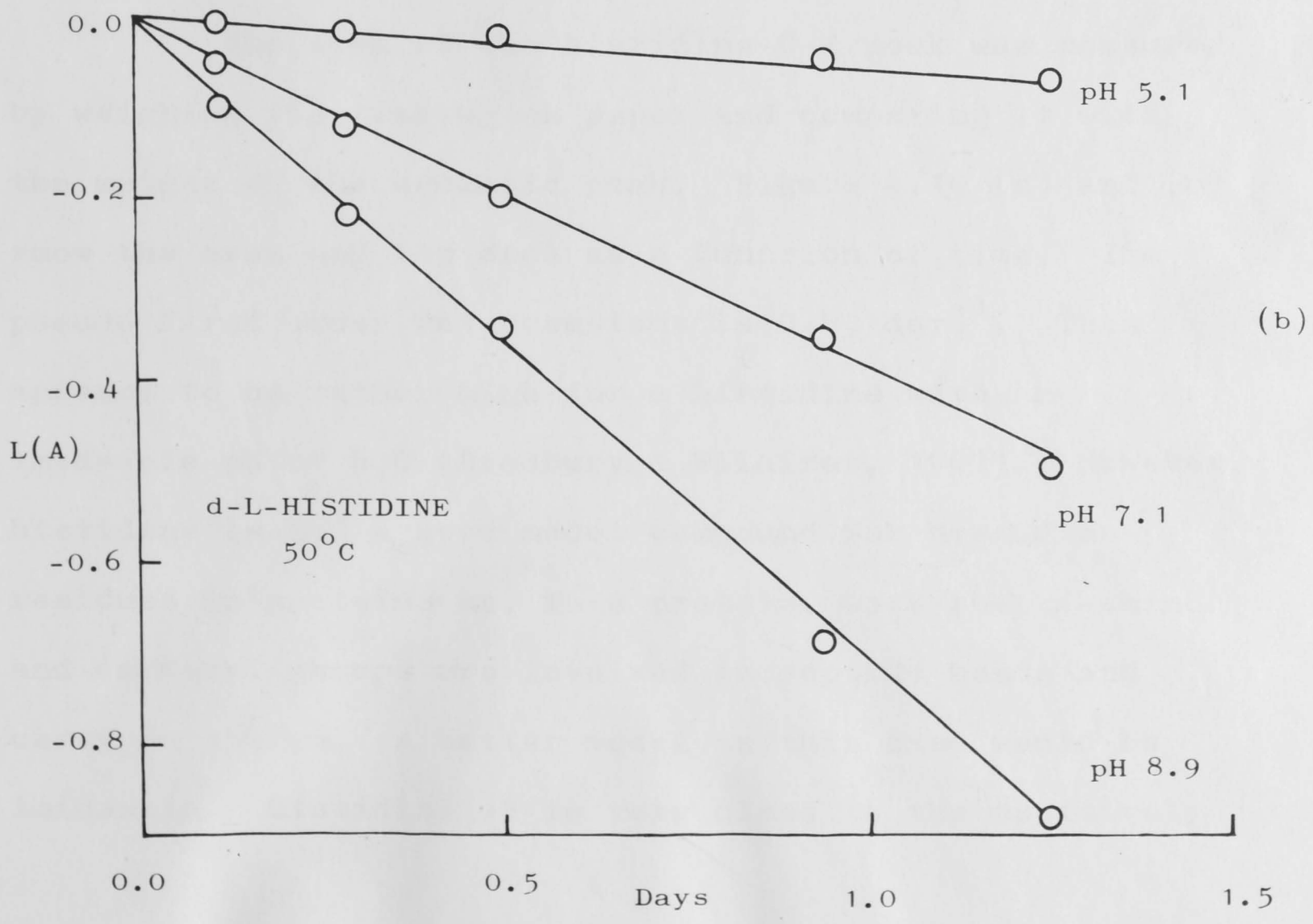
The influence of salt was determined by measuring the rate of exchange at 50°C in 0.10 M NaCl, as shown in Figure 4.8 (a). The open circles represent results for experiments with salt and the full circles represent results obtained without salt. The results are within experimental error of each other indicating that salt, at 0.10 M concentration, has no influence on the rate of reaction. Values of k_{obs} for the above experiments, as well as for some other experiments, are given in Table 4.4. Sodium acetate (0.10 M) has no effect on the rate showing that general base catalysis does not occur, and the rate of exchange in d-trifluoroacetic acid is zero as expected from the mechanism of exchange.

The back reaction was also studied. L-histidine was completely deuterated by leaving a sample in D_2O at pH 12 in a 37°C waterbath for seven days. The sample was lyophilised and the treatment repeated. The deuterated L-histidine was dissolved in water and the rate of protonation was determined at 50°C for three pH values. Values of k_{obs} were obtained from the slope of the lines in the semi-log plot shown in Figure 4.8 (b). The pseudo first order rate constant was found to be slightly lower than for deuteration due to the greater stability of the C-D bond compared to the C-H bond. If deuterated compounds are placed in water the temperature of the solution must be kept low to prevent the back exchange from occuring.

The rate of deuteration was also studied in a protein. Lysozyme was chosen as it has a single histidine

FIGURE 4.8


(a)
2.0
1.5
1.0
0.5
0.0
k_{obs}
L-HISTIDINE
50°C
0.1 M NaCl/D_2O
D_2O
0
2
4
6
8
10
12
pD


(b)
0.0
-0.2
-0.4
-0.6
-0.8
L(A)
d-L-HISTIDINE
50°C
pH 5.1
pH 7.1
pH 8.9
0.0
0.5
1.0
1.5
Days

residue, at position 15, that is clearly visible in the NMR spectrum at pH 2.8. A pH of 9 and a temperature of 37°C were chosen as these conditions, although they are fairly mild, give a reasonably rapid rate of deuteration. A 1% solution of the protein was made up in D_2O and treated under these conditions. At appropriate intervals some of the sample was removed and the solvent lyophilised off. As lysozyme aggregates above pH 7 causing the histidine C-2 peak to broaden into the baseline, samples of the deuterated protein were made up at 10% concentration in D_2O at pH 2.8. NMR spectra were obtained at 60 MHz except for the three days deuterated sample where the spectrum was obtained at 100 MHz. Figure 4.9 shows the aromatic region of lysozyme at 60 MHz for a number of samples. The spectra show that the C-2 peak diminishes in size with time.

The area of the histidine C-2 peak was measured by weighing its tracing on paper and comparing it with the weight of the aromatic peak. Figure 4.10 (a) and (b) show the area and log area as a function of time. The pseudo first order rate constant is 0.83 day^{-1}. This appears to be rather high for a histidine with an imidazole pK of 6.0 (Bradbury & Wilairat, 1967). However, histidine is not a good model compound for histidine residues in proteins as, in a protein, both the α-amino and carboxyl groups are involved in peptide bonds and carry no charge. A better model in this case would be imidazole. Histidine 15 is very close to the positively

FIGURE 4.9

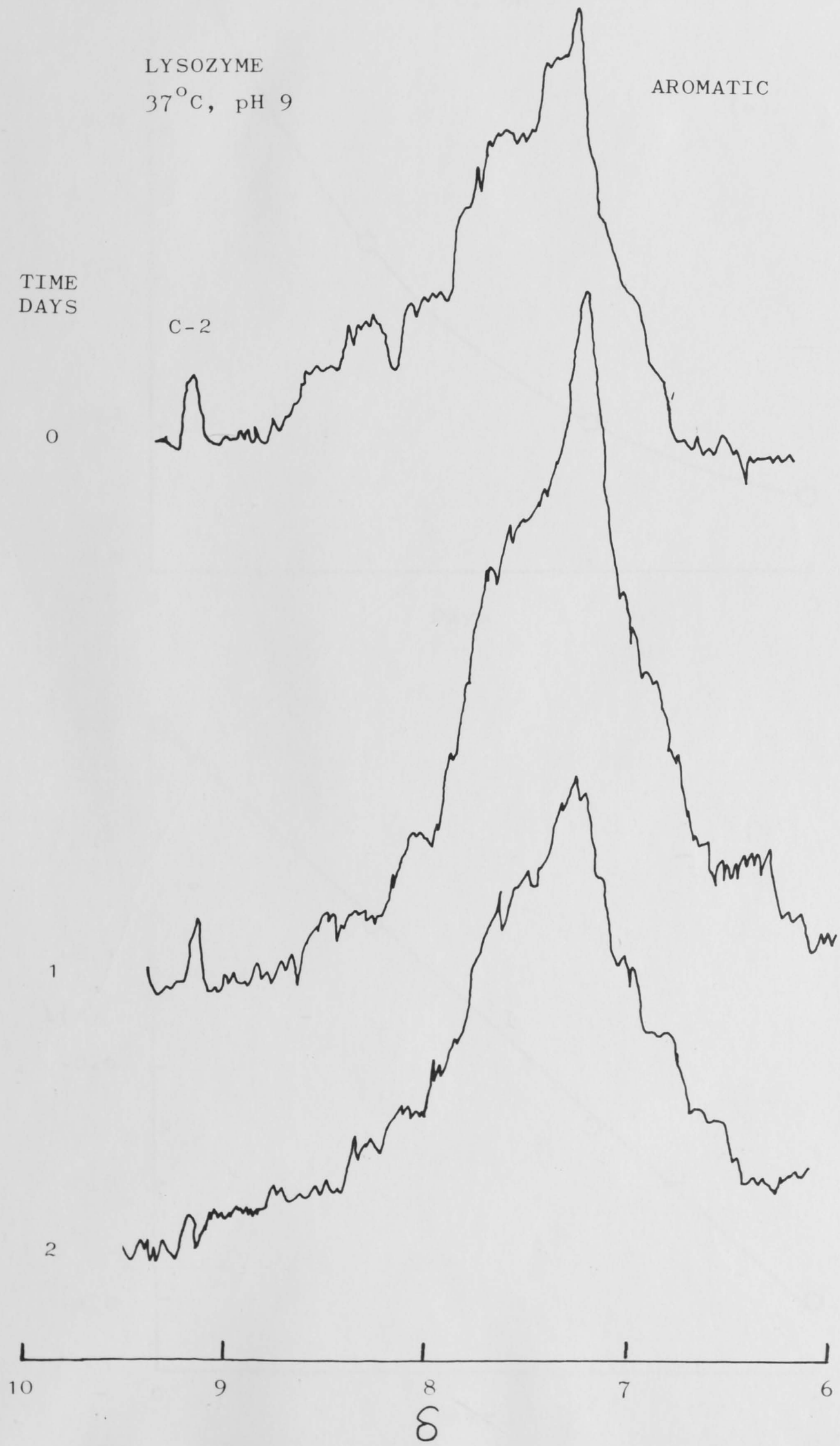
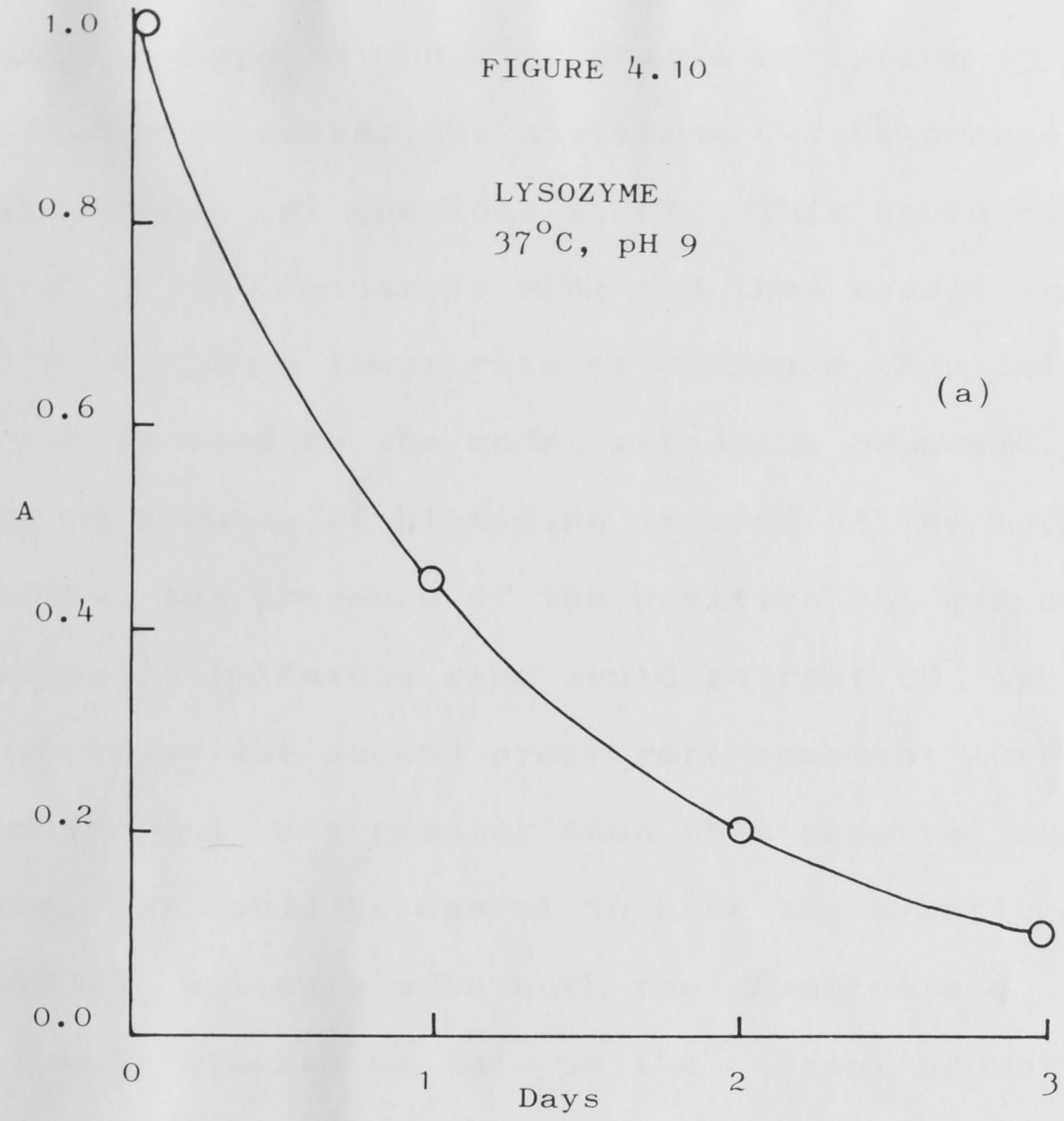
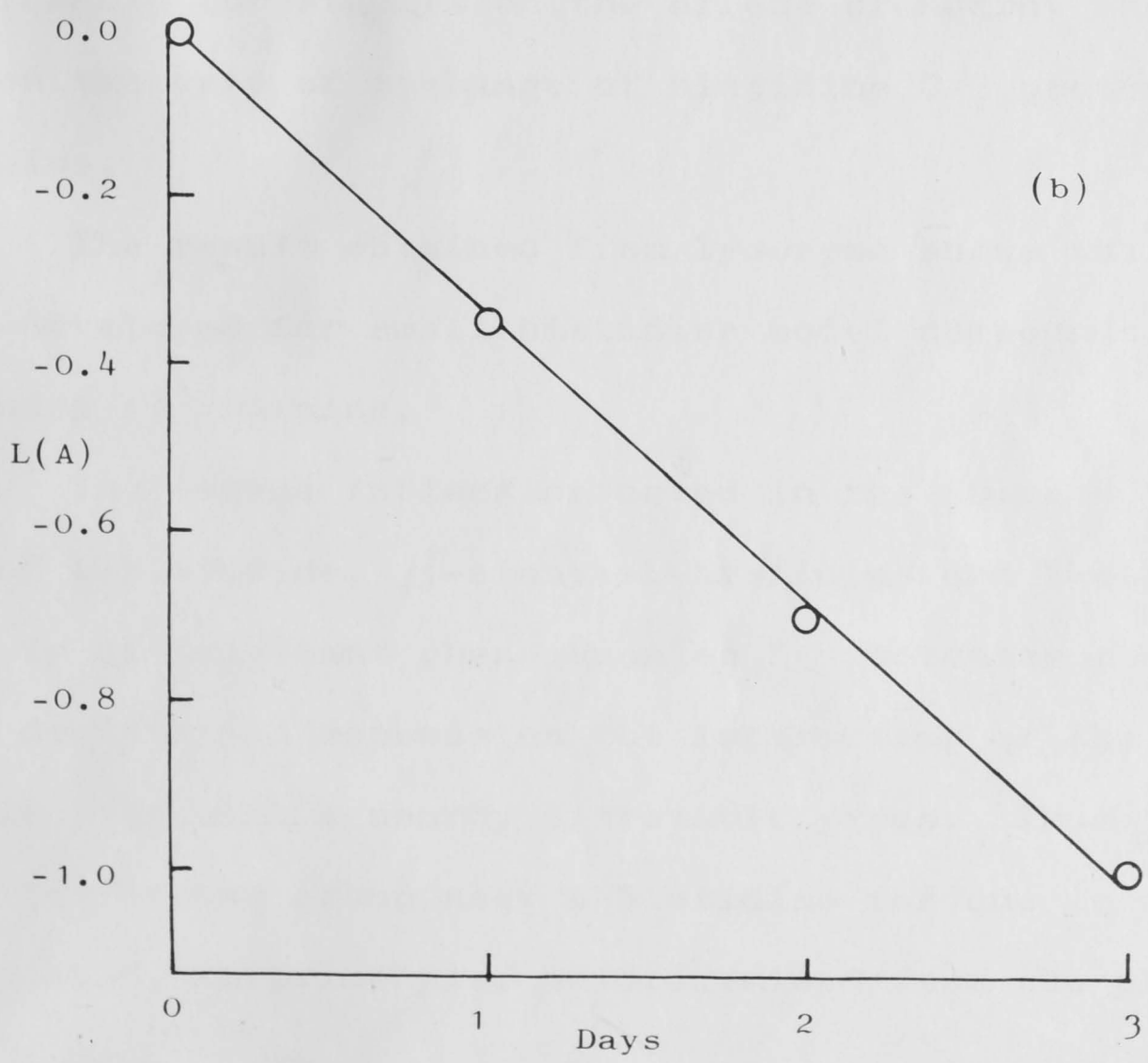


FIGURE 4.10

LYSOZYME
37°C, pH 9





charged amino groups on the side chains of lysine 13 and arginine 14, which causes the histidine C-2 resonance to have an abnormally low chemical shift. This would also lower the pK of the imidazole ring and thus result in histidine 15 having a lower rate of exchange than imidazole, if imidazole is used as the model reference compound. It could also be argued, if histidine is used as the model compound, that the presence of the positive charges near the histidine 15 imidazole ring would attract OD^- ions and thus increase the second order rate constant sufficiently to give an overall rate greater than that observed for L-histidine. It would be useful to have the kinetic data for a histidine molecule with both the α-amino and carboxyl groups blocked as this is the closest approximation to histidine residues in proteins, and would provide a good reference for studies on the effect of nearby charged groups on the rate of exchange of histidine C-2 protons in proteins.

The result obtained from lysozyme shows that the methods developed for small histidine model compounds can be extended to proteins.

The second inflection noted in the pD-rate curves of L-histidine, β-alanyl-L-histidine and L-histidyl-glycine is an important phenomenon as it indicates that the rate of deuteration depends on the interaction of the imidazole ring with a nearby titratable group. Thus the pK of a titratable group near a histidine residue in a protein could, in principle, be determined from the pD-rate

TABLE 4.4

The units of k_{obs} are $days^{-1}$.

L-histidine, 25°C.

pH	k_{obs}
5.0	0.00
7.1	0.05
9.0	0.08

L-histidine, 50°C.

pH	k_{obs}
5.0	0.13
7.0	1.22
8.9	2.05

L-histidine, 50°C

0.10 M NaCl

pH	k_{obs}
5.1	0.06
5.5	0.37
6.0	0.65
7.0	1.25
8.0	1.45
9.0	2.10

Protonation at 50°C of d-L-histidine

pH	k_{obs}
5.10	0.12
7.10	0.92
8.9	1.66

L-histidine in 0.10 M sodium acetate, 50°C.

pH	k_{obs}
7.2	1.30

Lysozyme, 50°C

pH	k_{obs}
9.0	0.83

profile for the exchange of the histidine C-2 proton.

A similar effect has been observed by Sachs et al (1971) for NMR measurements of chemical shift vs pH in L-histidine and histidine derivatives. The inflections arise in this system from changes in shielding around the C-2 proton when a titratable proton is removed at some distance from the ring. The kinetic results give a more prominent inflection than do NMR chemical shift measurements. Bradbury & Scheraga (1966) did not see these inflections when they titrated L-histidine across the pH range 1-13, as the removal of a proton from a nearby titratable group has only a small effect on the shielding and chemical shift of the C-2 proton. Although the inflection in the chemical shift curve for the C-4 proton is more prominent than for the C-2 proton, it is smaller than that observed in the kinetic plots. It would be unlikely that an inflection would be seen in the chemical shift curve of β-alanyl-L-histidine above neutral pH due to the removal of the amino proton.

Chemical shift measurements, however, have some advantages over kinetic measurements. They are easier to carry out and can be used to determine the pK of titratable groups at acid pH where there is no exchange reaction. Kinetic measurements are more sensitive and proteins may be deuterated at any desired concentration, thus minimising any aggregation that may take place. For example lysozyme aggregates above pH 7 and the C-2 histidine resonance can not be seen in the NMR spectrum. In this case

chemical shift measurements could not be used to see if there was a group, titrating at alkali pH, near the histidine residue. However, the protein could be deuterated at alkali pH at low concentration, where aggregation would be minimised. The deuterated protein could then be isolated by lyophilisation and the area of the C-2 resonance measured at pH 2.8 at a 10% concentration of the protein. In this manner a curve of k_{obs} vs pH could be constructed over a wide pH range. Titratable groups, near the histidine residue, could be detected by the presence of inflections in the rate curve.

The method appears to show some promise in the solution of certain problems in protein chemistry. The application of this method in the differential deuteration of the histidine C-2 protons in ribonuclease-A is given in Chapter 8.

Note: The work on the histidine C-2 proton exchange in ribonuclease-A has been placed at the end of the thesis as the experimental work was carried out while the rest of the thesis was being typed.

CHAPTER 5

THE AGGREGATION OF α-CHYMOTRYPSIN AND TRYPSIN

5 A INTRODUCTION

The resonances observed in the nuclear magnetic resonance spectra of macromolecules, and proteins in particular, are usually very broad compared with those of small molecules. This effect has been noted by a number of workers, Kowalsky (1962), McDonald & Phillips (1969) and Bradbury & King (1969). The resonance broadening observed in protein spectra arises from a number of sources and an explaination of this phenomenon has been given in Chapter 2. A considerable amount of effort has been made to eliminate broad and overlapping resonances by the use of hign magnetic fields to separate individual resonances, selective deuteration of specific amino acids in the protein to simplify spectra (Markley et al., 1968; Crespi & Katz, 1969) and difference spectroscopy (King & Bradbury, 1971).

On the other hand very little attention has been given to utilising this effect in the study of protein chemistry, although there is a great deal of information to be gained from such studies. The width of a peak will vary inversely with the relaxation times of the protons contributing to that peak, and thus can be used in the study of the aggregation and the mobility of specific groups in proteins as a function of change in their environment.

Bradbury & Wilairat (1967) first reported the absence of the histidine C-2 resonance in the spectra of α-chymotrypsin and trypsin in D_2O at 60 MHz. As trypsin is known to contain three histidine residues, two of which are at the active site, and α-chymotrypsin two, both at the active site, the authors suggested the absence of the resonance could be due to dipolar broadening caused by the high molecular weight of these proteins (≈24,000). Subsequent work by King (1970) at 100 MHz has shown the presence of one of the three possible histidine C-2 peaks in spectra of trypsin at pH 3.5. A spectrum was also obtained of α-chymotrypsin at pH 7 showing a peak that may be due to a histidine C-2 proton, although the assignment is not conclusive.

Although the aggregation of α-chymotrypsin has been the subject of a number of studies, the technique of NMR spectroscopy has not been used in any of them. Schwert (1949) and Schwert & Kaufman (1951) found that α-chymotrypsin underwent a concentration-pH dependent aggregation by measuring sedimentation and diffusion constants as a function of pH. Smith & Brown (1952) observed that diisopropylphosphoserine 195-α-chymotrypsin aggregated in the same manner as α-chymotrypsin and concluded that the active site is not involved in the aggregation reaction. Steiner (1954) was able to show, by using the technique of light scattering, that the reaction was a monomer-dimer equilibrium at pH 4 and that the presence of salt increased the aggregation. He also noted that variation in temperature had no effect on

the aggregation, indicating that electrostatic interactions are responsible for the association.

Egan et al (1957) reported there was no aggregation at pH 2.5 and maximum aggregation at pH 4.0. The photoxidation of histidine 57 caused the disappearance of any tendency to associate and the authors concluded that the active site is involved in the aggregation reaction, an opposite view to that taken by Smith & Brown (1952).

A number of workers, Tinoco (1957), Roa & Kegeles (1958) and Bethune & Kegeles (1961), found that, at pH 6, α-chymotrypsin undergoes further association to give trimers. Very little is known about the mechanism of this reaction. Above pH 7, in the presence of 0.10 M NaCl, Tinoco (1957) and Krigbaum & Godwin (1968) have shown that α-chymotrypsin is monomeric.

Neet & Brydon (1970) found that the presence of small groups, such as acetyl, had no effect on aggregation in agreement with Moromito & Kegeles (1967), who concluded that the active site was not involved in the association. The presence of large groups, such as tosyl, eliminated aggregation at pH 4 but not at pH 6. Neet & Brydon (1970) stated that the active site of α-chymotrypsin was involved in the association but that small groups, such as acetyl or diisopropylphospho, do not alter the active site structure sufficiently to stop aggregation.

Cunningham et al (1953) reported that trypsin shows a concentration dependent monomer-polymer equilibrium above pH 7 in 0.01 M $CaCl_2$ solutions. Below pH 7 the enzyme

gives the characteristics of a monodisperse solution, as do the zymogen and diisopropylphospho-serine 185-trypsin throughout the pH range. The authors stated that the active site appeared to be involved in the association reaction.

In this chapter broadening of resonances has been used to follow aggregation in α-chymotrypsin and trypsin.

5 B EXPERIMENTAL

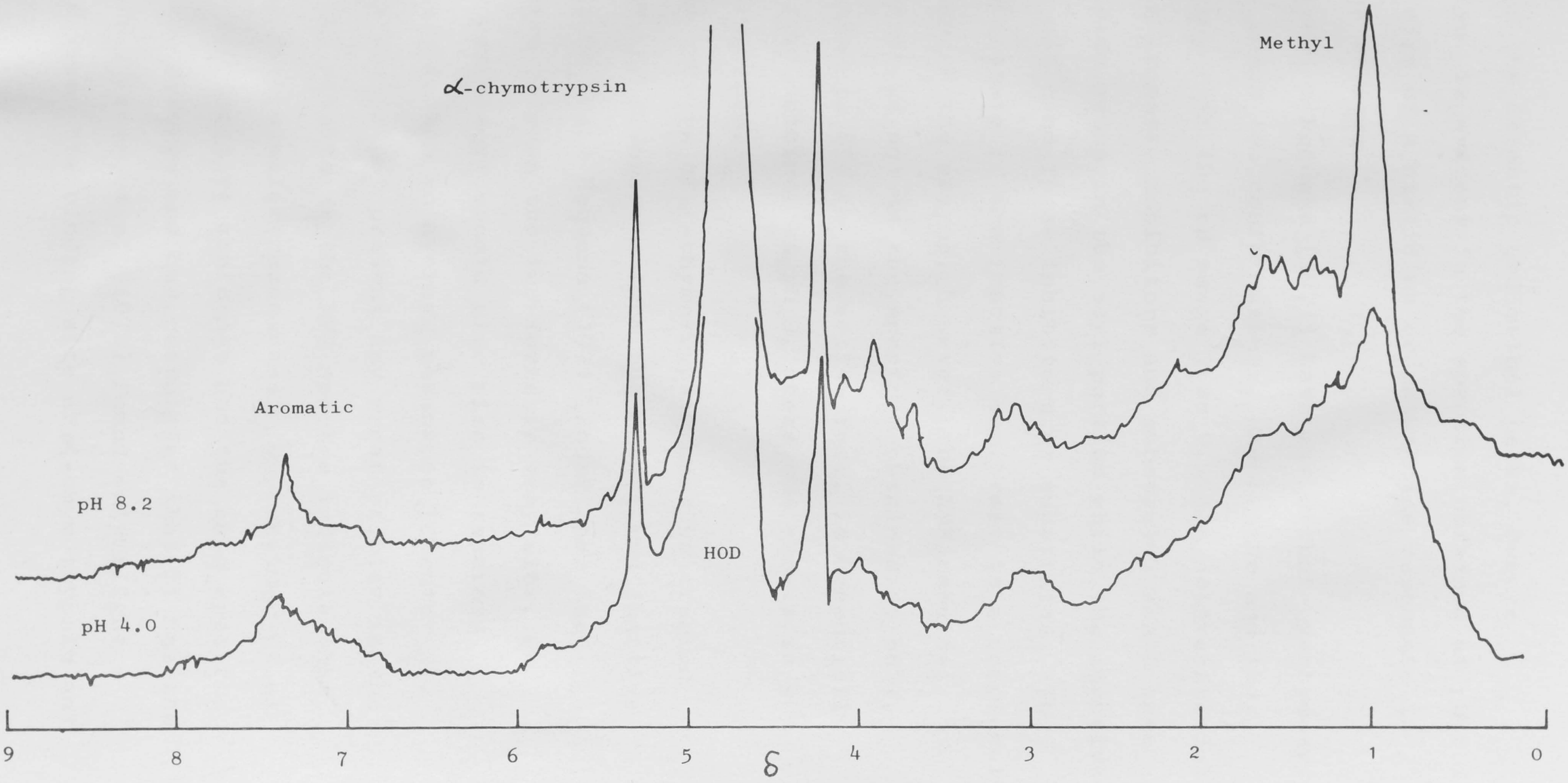
Solutions of α-chymotrypsin and its derivatives were made up at 10% concentration (w/v) in 0.10 M NaCl in D_2O unless otherwise stated. Trypsin solutions were made up at 10% concentration in 0.01 M $CaCl_2$ in D_2O. The pH was adjusted by the addition of 6 M HCl/D_2O or 6 M NaOD and was measured at the end of each experiment. Spectra were accumulated for three to sixteen hours on a Perkin Elmer 60 MHz or Varian 100 MHz spectrometer. Some spectra were also obtained on a Varian 220 MHz spectrometer.

Widths of the aromatic peak, consisting of resonances from phenylalanine, tyrosine, tryptophan and C-4 histidine protons, and the methyl peak, consisting of resonances from leucine, isoleucine and valine methyl protons, were measured at half peak height and are given in parts per million to allow the placement of results obtained at 60 and 100 MHz on the same graph.

5 C RESULTS

Spectra of Worthington α-chymotrypsin at 60 MHz in 0.10 M NaCl in D_2O at pH 4.0 and 8.2 are shown in Figure 5.1.

Figure 5.1



Sharpening of the aromatic and methyl peaks, due to disaggregation, is evident in the spectrum obtained at pH 8.2. There is no sign of a histidine or methionine resonance in either of the spectra.

Fersht & Requena (1971) have shown that α-chymotrypsin exists in at least two conformations, one active and the other inactive, over the pH range 3 to 9. The active species binds certain aromatic inhibitors and substrates which have an aromatic side group in the acyl portion while the inactive species does not bind these inhibitors or substrates. The authors do not state if the inactive conformer is a completely denatured form of the enzyme, however, the NMR results indicate that it is not as the spectrum obtained at pH 7, where the enzyme is 85% in the active form, is essentially the same as that obtained at pH 9, where the enzyme is 90% in the inactive form.

The spectra of α-chymotrypsin must be treated as the sum of the resonances from the active and inactive conformers as Fersht & Requena (1971) found that the interconversion between the two forms is very slow, and thus the two conformers should give rise to separate resonances rather than an average resonance (Chapter 2). However, this should not present any great problem in the analysis of the results as the NMR spectra indicate that the two forms give similar resonances. Oppenheimer et al (1966) found the inactive conformer had the same specific rotation as the zymogen and has postulated that it has the same structure. Freer et al (1970) found only slight differences between the binding site of α-chymotrypsin and

the same region in the zymogen where a section of polypeptide chain lies across the binding site. Fresht & Requena (1971) have postulated that the same section of the poylpeptide chain lies across the binding site of the inactive α-chymotrypsin. The assumption that only small differences exist between the two conformers appears fairly reasonable.

As α-chymotrypsin is known to autolyse at pH 6-10 (Kumar & Hem, 1970), experiments were carried out to determine the extent of autolysis and its effect on the NMR spectrum. The enzyme is known to be stable to autolysis at pH 4 so a spectrum was obtained at pH 4 and used as a reference. Solutions of the enzyme were kept at pH 6, 9 and 11.5 for various periods of time and then readjusted to pH 4. As autolysis would result in the breaking of peptide bonds and increased mobility of groups within the enzyme, its effect would be seen in the sharpening of peaks in the NMR spectrum obtained at pH 4. The spectra of solutions readjusted to pH 4 were obtained and compared with the reference spectrum to determine if there were any differences in the peaks.

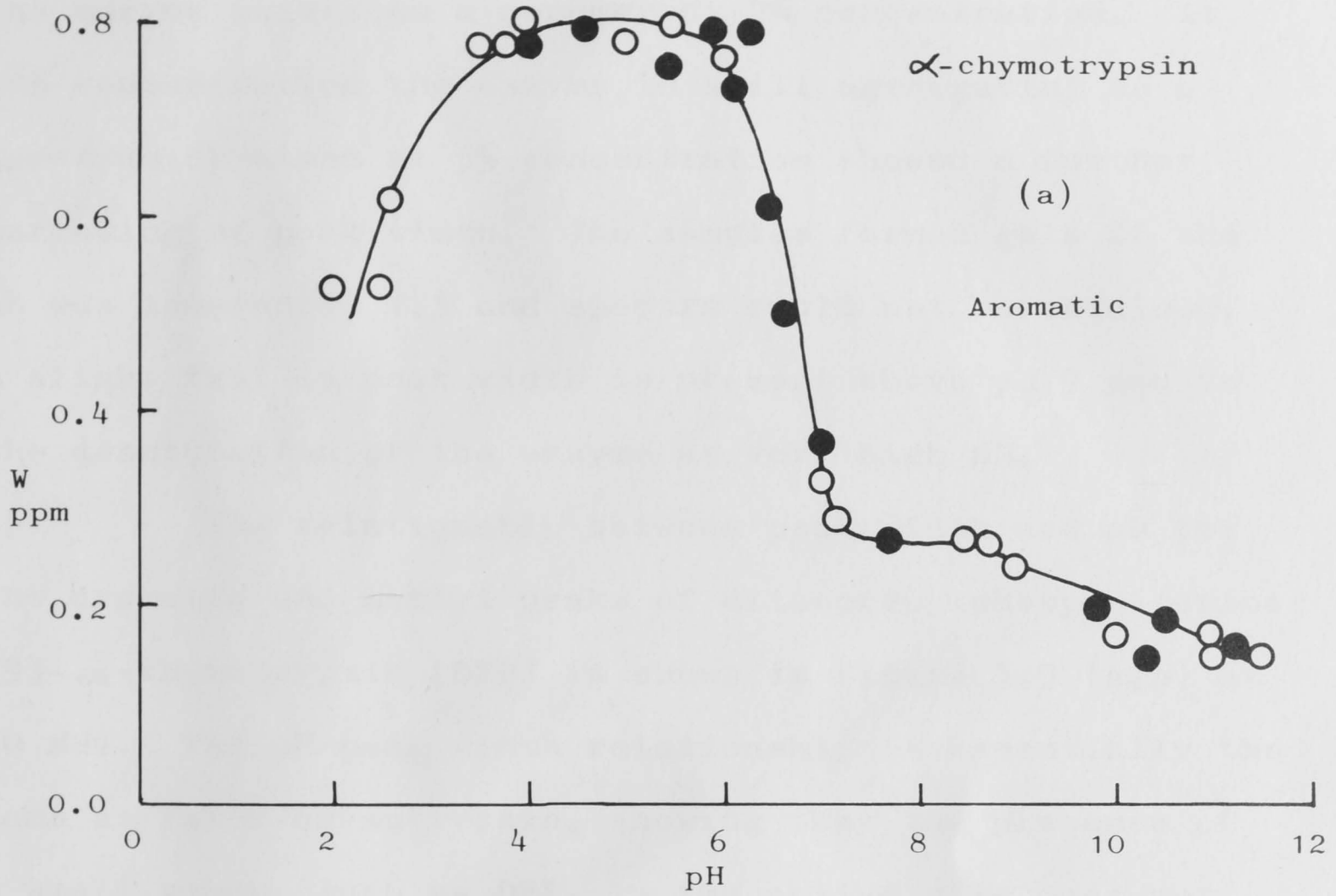
Solutions kept at pH 6 for periods of up to six hours gave identical spectra at pH 4 to that of the reference indicating that autolysis is very slow at this pH. If solutions were kept at pH 6 for twenty four hours a sharpening of the methyl peak was noticable. Spectra from samples kept at pH 9 for three hours showed a slight sharpening of the methyl peak similar to that observed in spectra of samples kept at pH 6 for twenty four hours. The pH of

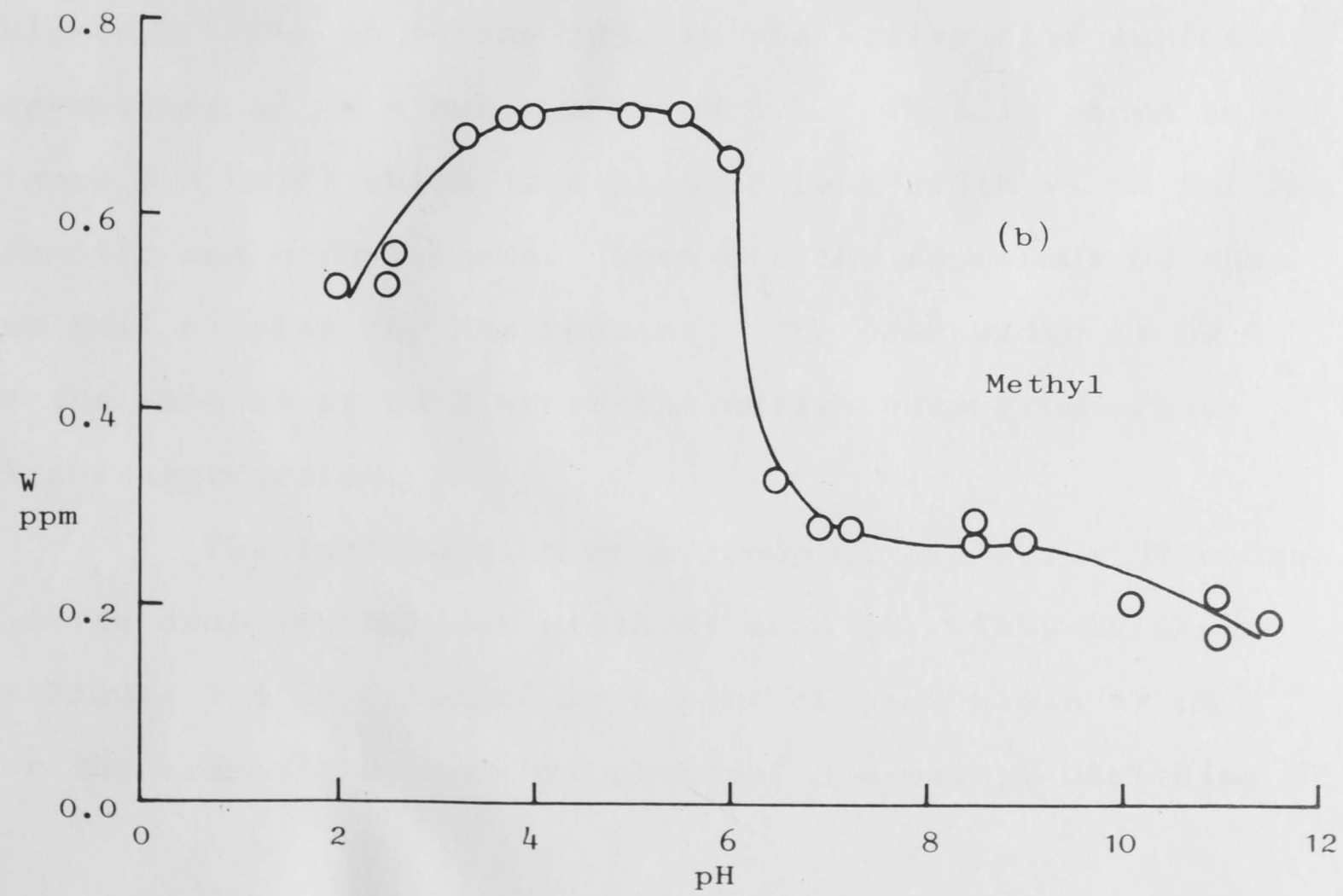
solutions kept at pH 9 for three hours dropped by 0.10 pH units. The above observations indicate that a small amount of autolysis is taking place at pH 9. If samples were left for longer periods of time at pH 9, sharpening of all peaks was very prominent due to the extensive autolysis taking place. The spectrum of a sample left at pH 11.5 for three hours showed extreme sharpening of all peaks, as the spectrum obtained at pH 4 was identical to that obtained at pH 11.5. This sharpening must be due to an irreversible denaturation of the enzyme as it does not autolyse at this high pH.

It can be assumed, from these results, that autolysis taking place over the pH range 6 to 9 is not having a marked effect on the shape of spectra obtained by accumulation over three hours or less, so the spectra can be regarded as being representative of the native enzyme.

The width at half peak height of the aromatic and methyl peaks is plotted as a function of pH in Figure 5.2 (a, b). Open circles represent widths obtained at 60 MHz and full circles represent widths obtained at 100 MHz. Krigbaum & Godwin (1968) found that α-chymotrypsin was monomeric at 6% protein concentration above pH 7 in 0.10 M NaCl in D_2O. A spectrum was obtained under these conditions at pH 7 and it was found to have the same peak widths as the spectrum obtained at 10% protein concentration. This indicates that the enzyme is also monomeric at the higher concentration. There is a large increase in width between pH 3 and 6.5 due to the aggregation of the enzyme. Below

Figure 5.2


0.8
0.6
0.4
W
ppm
0.2
0.0
0
2
4
6
8
10
12
pH
∝-chymotrypsin
(a)
Aromatic


0.8
0.6
0.4
W
ppm
0.2
0.0
0
2
4
6
8
10
12
pH
(b)
Methyl

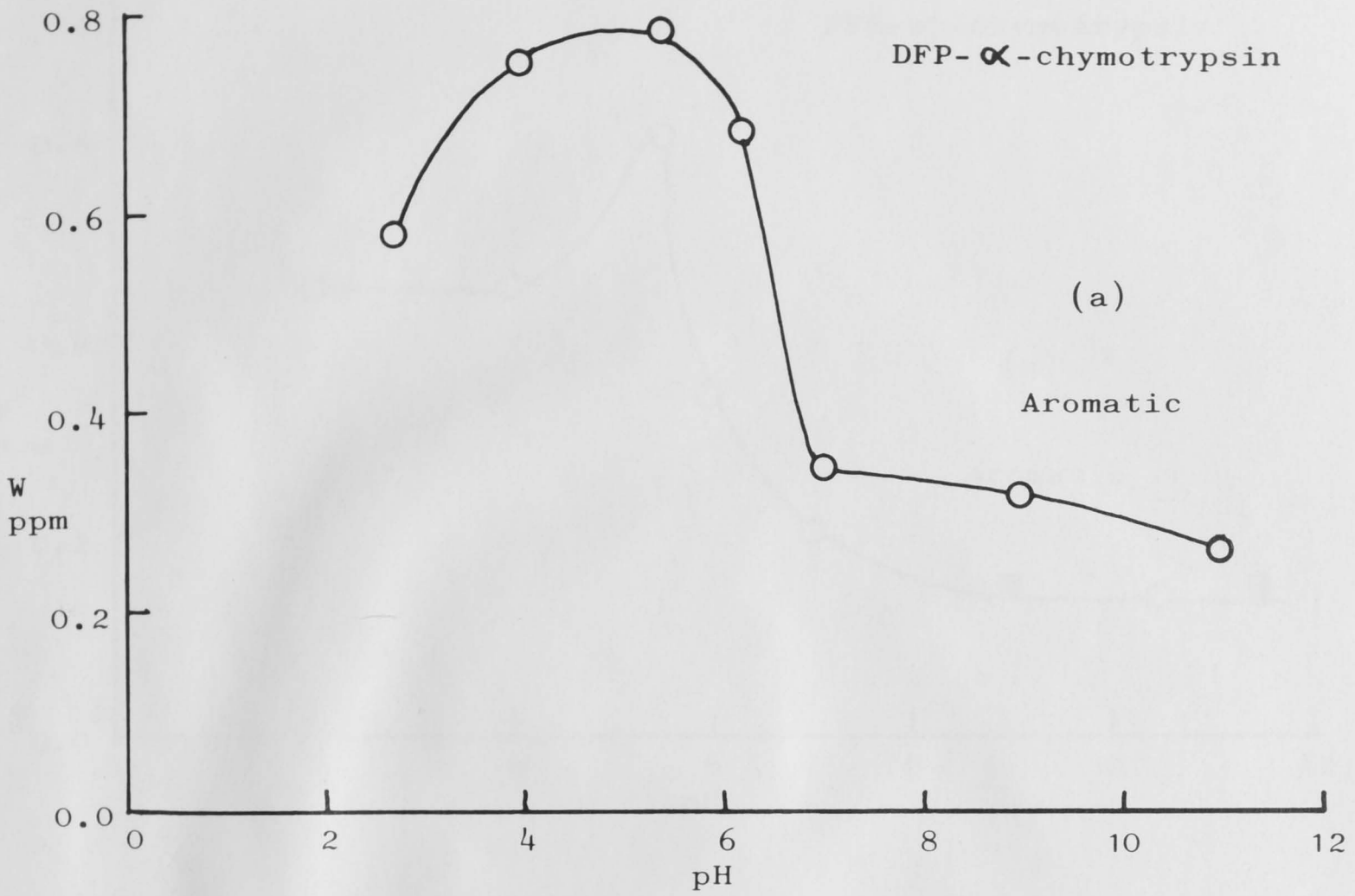
pH 3 there is a narrowing in the peak width which reaches a minimum value at pH 2.5 where Egan et al (1957) found the enzyme exists as a monomer at 2% concentration. At 10% concentration the enzyme is still aggregating as a spectrum obtained at 5% concentration showed a further narrowing of peak width. The samples formed gels if the pH was lowered to 1.5 and spectra could not be obtained. A slight fall in peak width is present above pH 9 due to the denaturation of the enzyme at very high pH.

The relationship between peak width and pH for the aromatic and methyl peaks of diisopropylphospho-serine 195-α-chymotrypsin (DFP) is shown in Figure 5.3 (a,b) at 60 MHz. The pH-peak width relationship is essentially the same as for α-chymotrypsin, showing that the presence of a small group, such as DFP, in the active site does not affect the aggregation reaction.

The presence of a large group, p-fluorophenyl-sulphonyl (FPS) on serine 195, in the active site inhibits aggregation at pH 4 but not at pH 5.5. This is shown in Figure 5.4 (a,b) which is a plot of peak width vs pH for the aromatic and methyl peaks. Open circles represent 60 MHz and full circles 100 MHz results. The peak width at pH 4 is the same as at pH 2 where the native enzyme undergoes slight aggregation.

The introduction of a group at histidine 57 causes a large drop in the peak width at acid pH. This is shown in Figure 5.5 (a,b) which is a plot of peak width vs pH for the aromatic and methyl peaks of 3-N-methyl-histidine 57

Figure 5.3


0.8
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
12
pH
DFP-∝-chymotrypsin
(a)
Aromatic

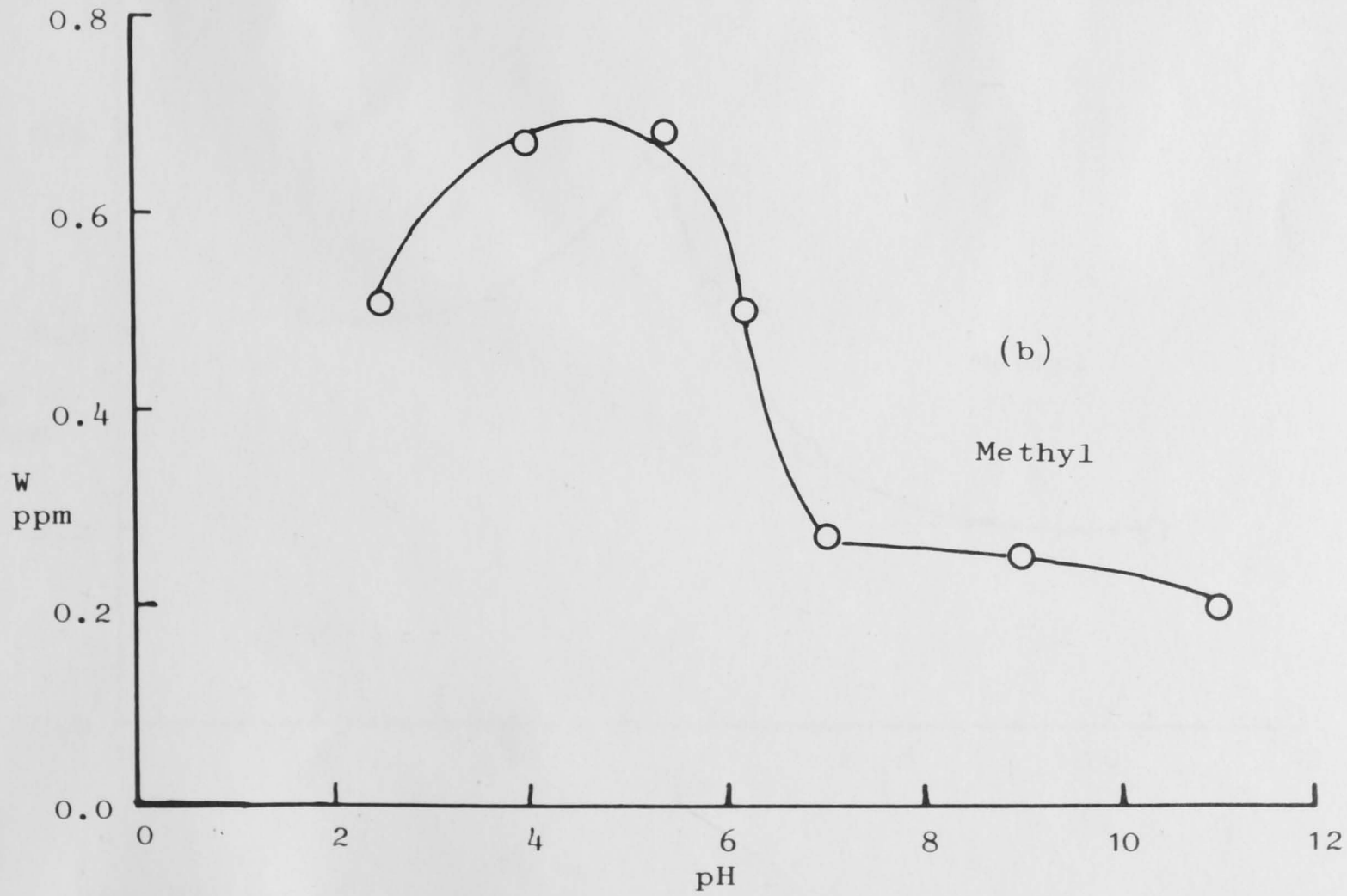
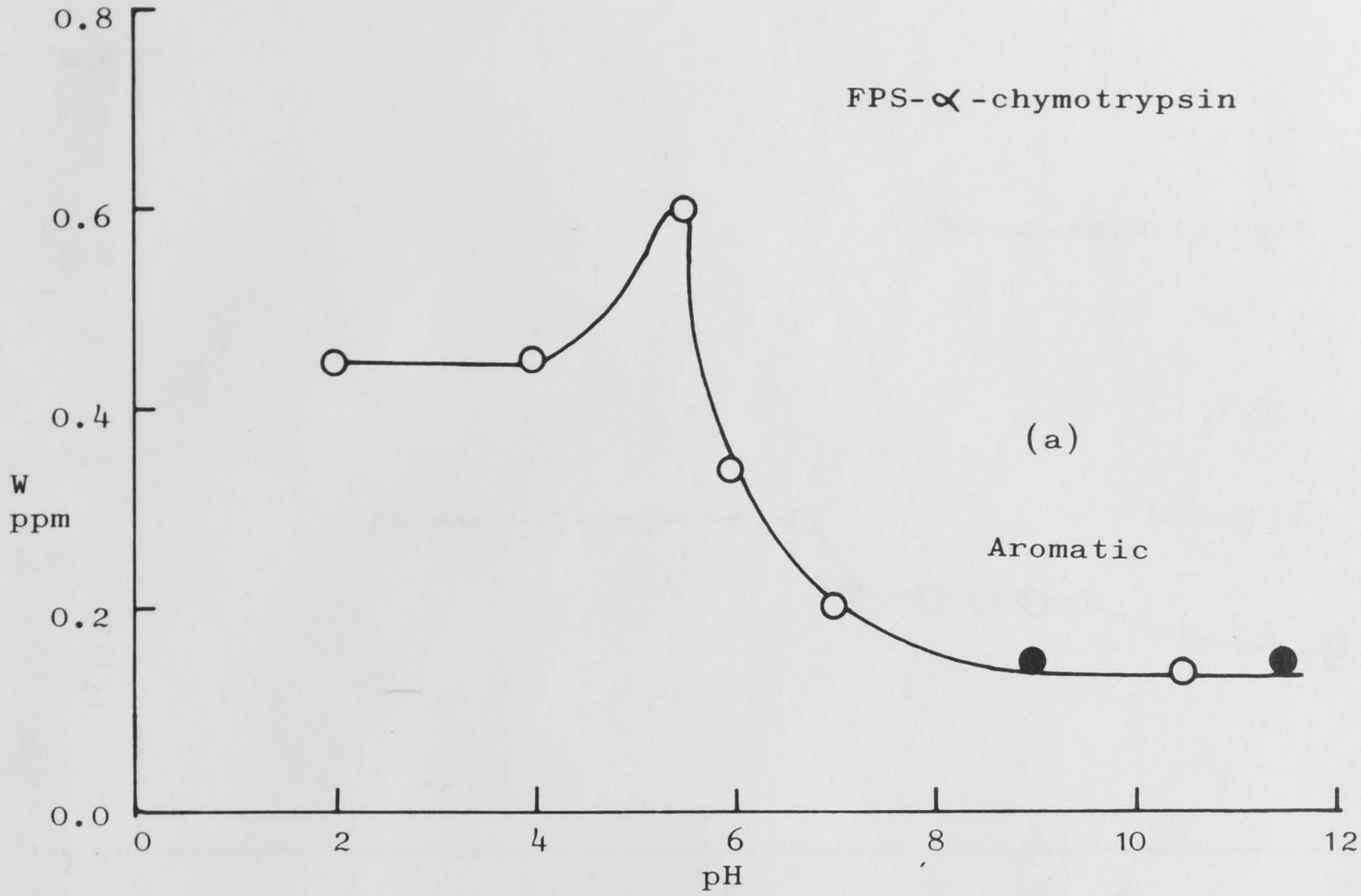

0.8
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
12
pH
(b)
Methyl

Figure 5.4


FPS-∝-chymotrypsin
(a)
Aromatic
W
ppm
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
pH

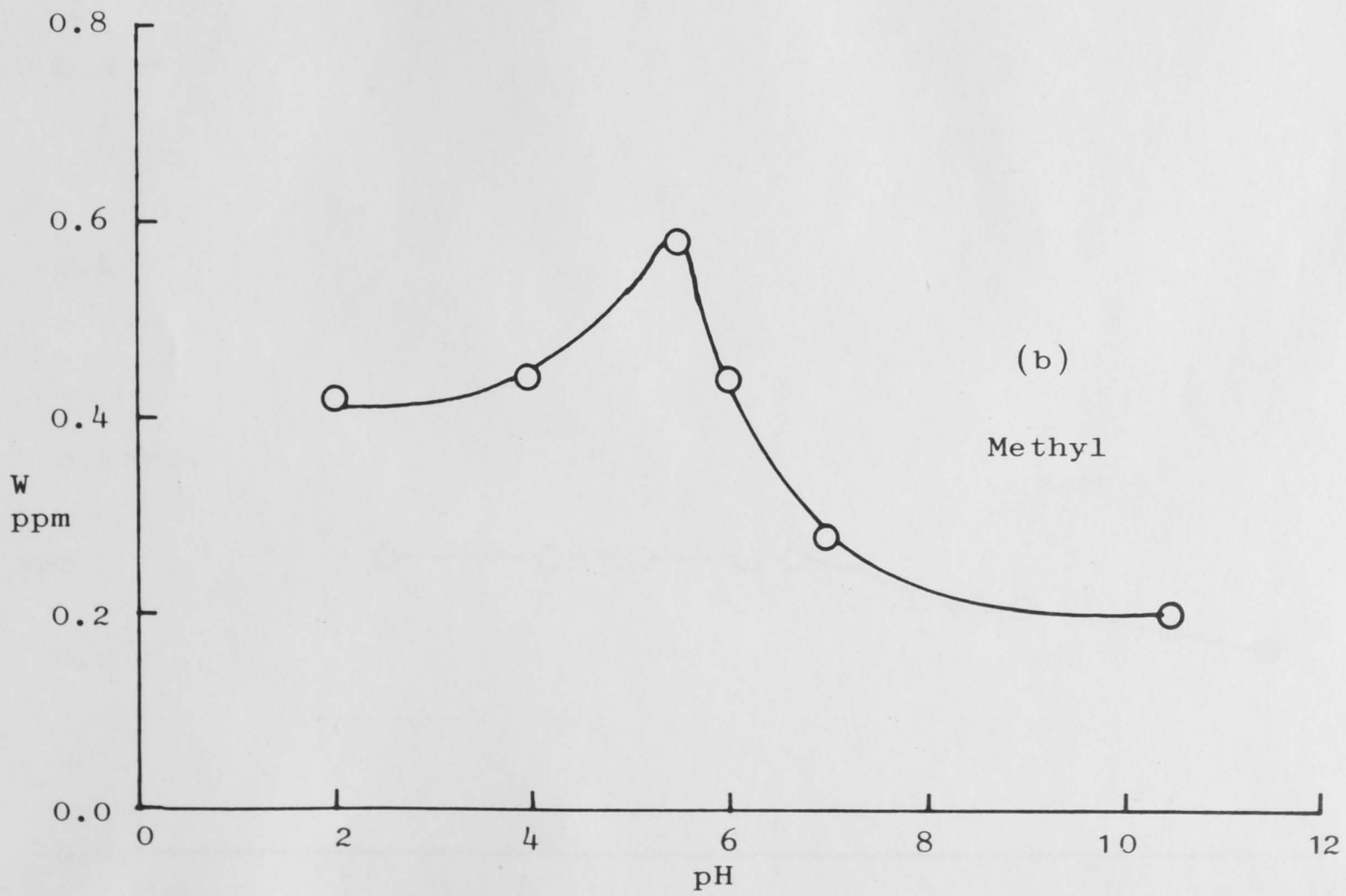
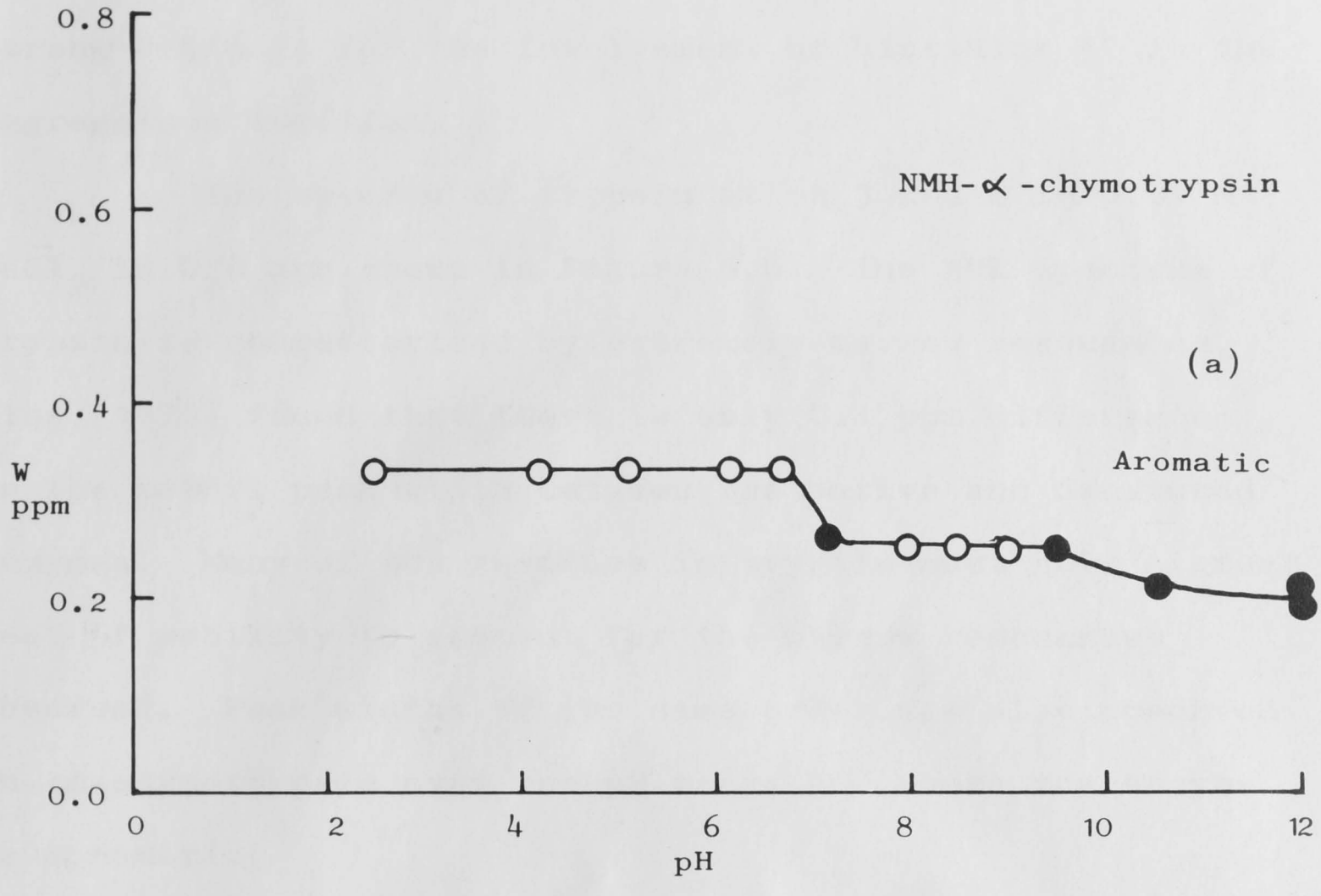
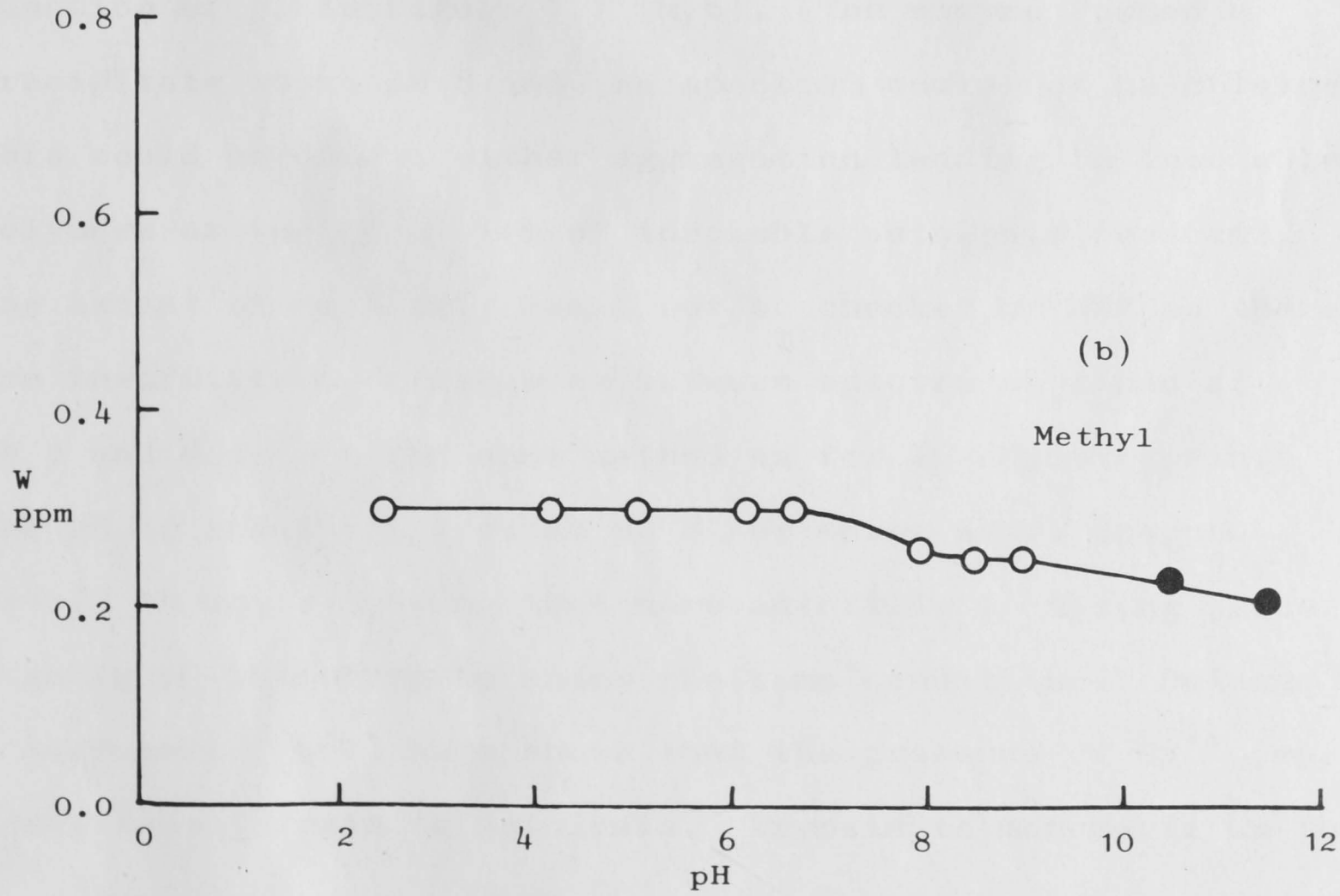

(b)
Methyl
W
ppm
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
pH

Figure 5.5


0.8
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
12
pH
NMH-∝-chymotrypsin
(a)
Aromatic


0.8
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
12
pH
(b)
Methyl

-α-chymotrypsin (NMH). The peak width at acid pH is only slightly larger than at alkali pH. This result provides strong evidence for the involvement of histidine 57 in the aggregation reaction.

The spectra of trypsin at pH 3 and 8 in 0.01 M $CaCl_2$ in D_2O are shown in Figure 5.6. The NMR spectrum of trypsin is characterised by extremely narrow resonances. King (1970) found that there is only 0.1 ppm difference in the methyl peak width between the native and denatured enzymes. Many of the residues in trypsin must have a great deal of mobility to account for the narrow resonances observed. Peak widths of the same order are also observed in α-chymotrypsin over the pH range 7-9 where the enzyme is monomeric.

The width of the aromatic and methyl peaks of trypsin in 0.01 M $CaCl_2$ in D_2O at 60 MHz is plotted as a function of pH in Figure 5.7 (a,b). The enzyme formed a precipitate above pH 8 and the spectrum could not be obtained. This could be due to either aggregation leading to insoluble polymers or the formation of insoluble autolysis products. The extent of autolysis could not be checked by NMR as there are insufficient differences between spectra obtained at pH 3 and 8 to use the same method as for α-chymotrypsin. The pH of solutions kept at pH 8 for three hours dropped by 0.2 pH units showing that more autolysis is taking place than in α-chymotrypsin under the same conditions. Delaage & Lazdunski (1967) have shown that the presence of Ca^{++} ions stabilises trypsin to autolysis. Trypsin is monomeric up to

Figure 5.6

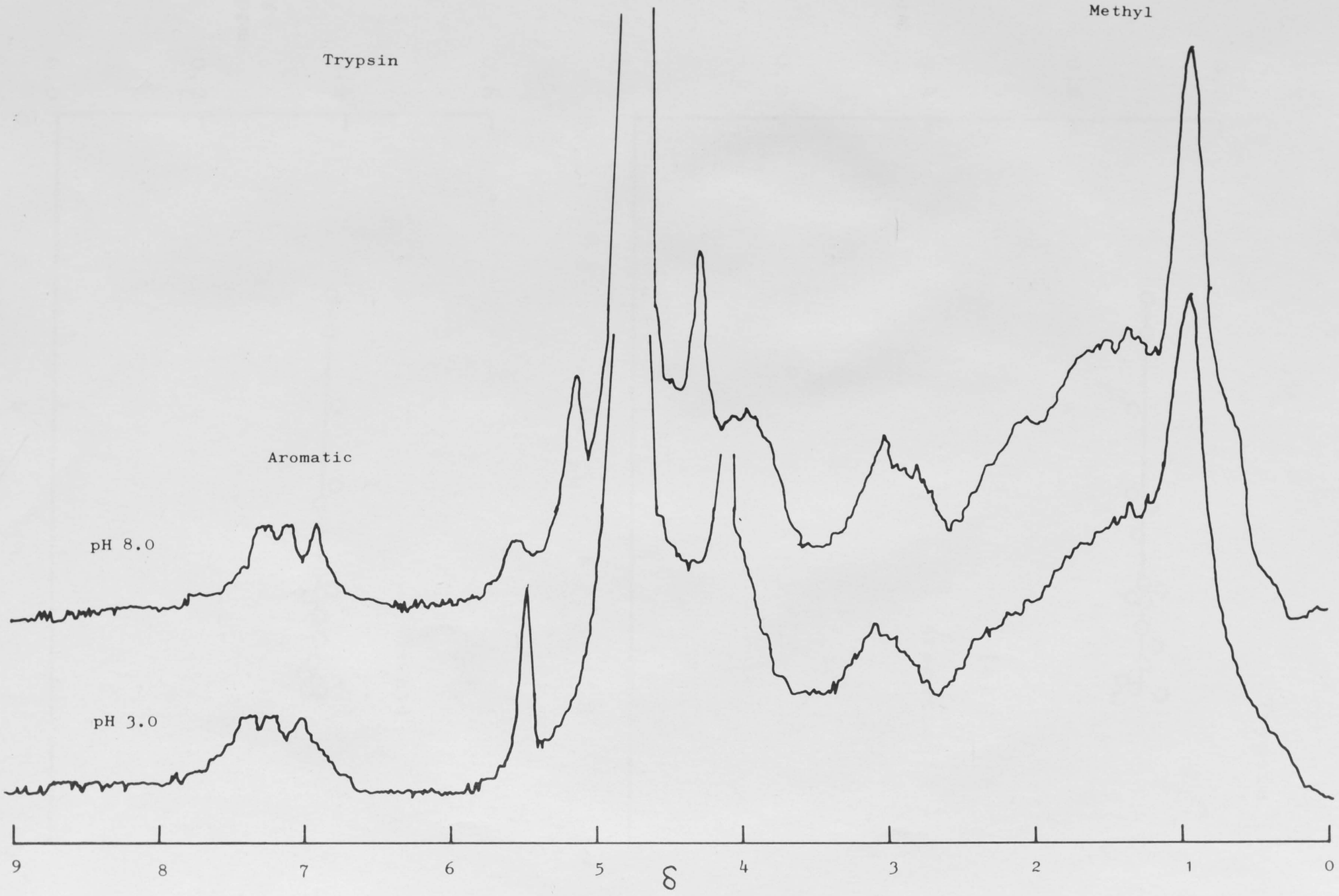
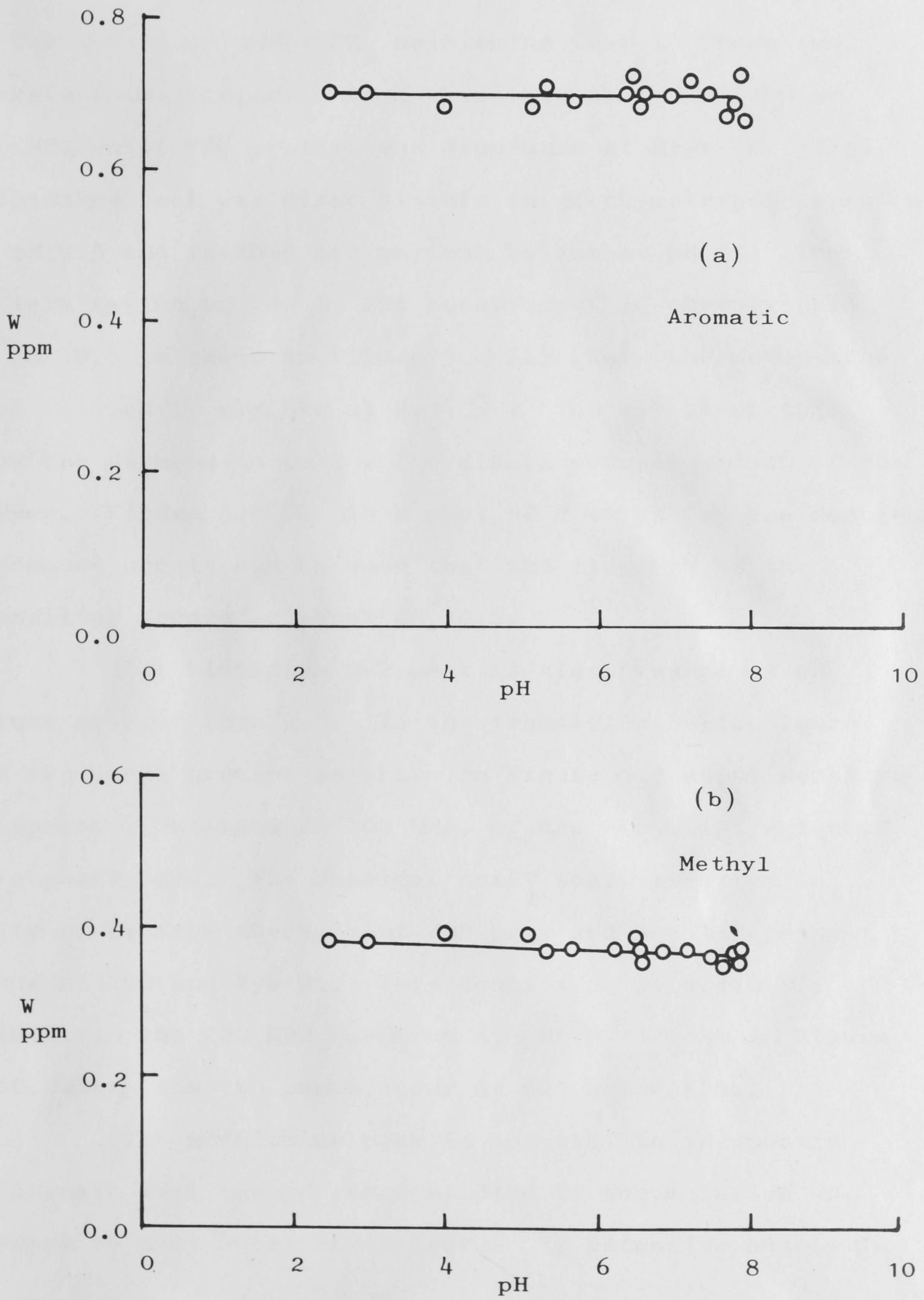


Figure 5.7


Trypsin
0.8
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
pH
(a)
Aromatic
0.6
0.4
0.2
0.0
W
ppm
0
2
4
6
8
10
pH
(b)
Methyl

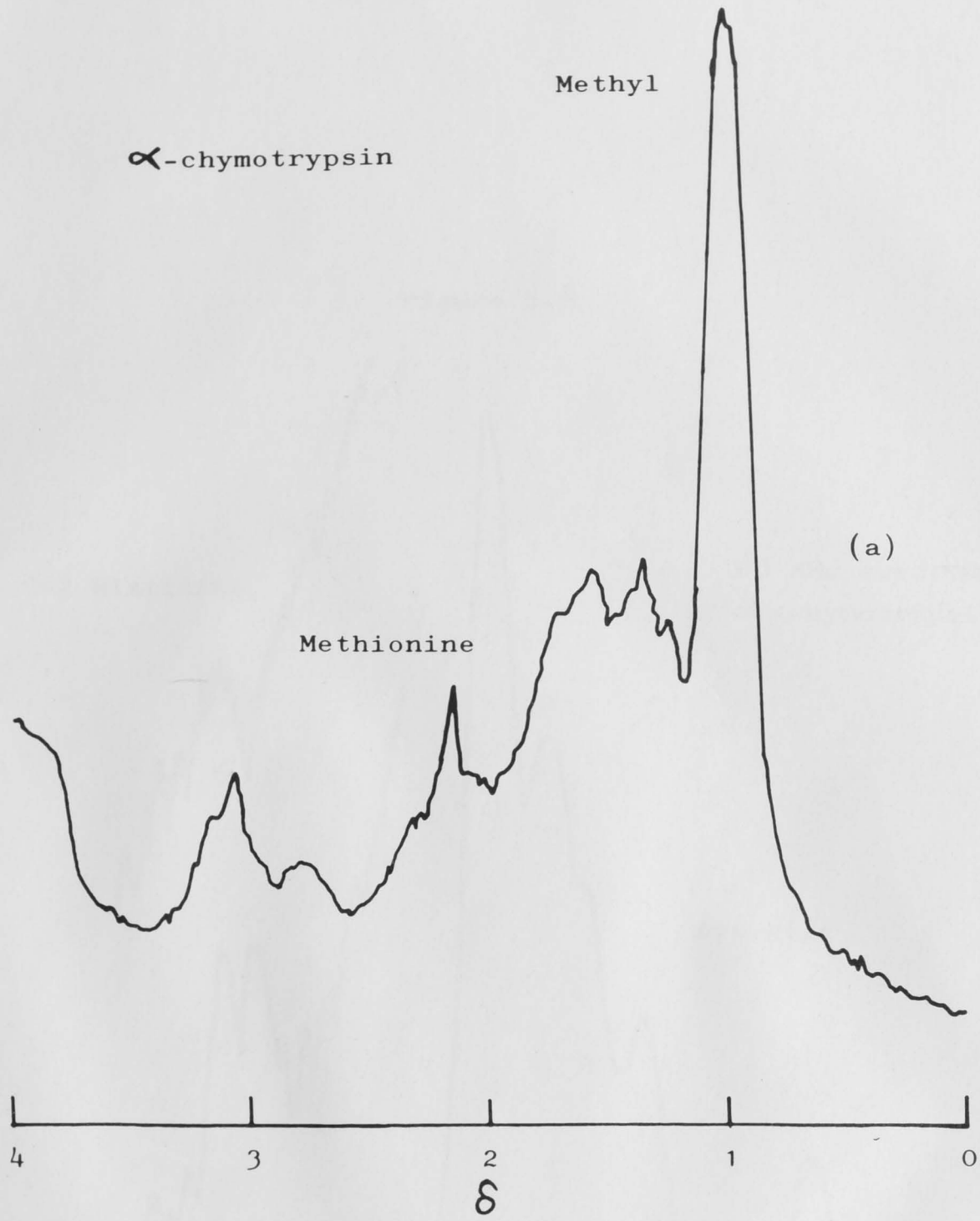
pH 8 as the widths of the aromatic and methyl peaks are fairly constant over the pH range 2.5 to 8.0. The slight downward trend with increasing pH could be due to autolysis.

All spectra were closely examined for the presence of C-2 histidine and $-SCH_3$ methionine peaks. These two peaks were not observed in α-chymotrypsin at 60, 100 or 220 MHz until the protein was denatured at high pH. The methionine peak was first visible in α-chymotrypsin spectra at pH 9.5 and reached its maximum height at pH 11. The upfield region of the 60 MHz spectrum of α-chymotrypsin at pH 10.5 is shown in Figure 5.8 (a) where the methionine peak is clearly visible at 2.14 δ . The height of this peak can be used to follow the alkaline denaturation of the enzyme. Figure 5.8 (b) is a plot of F vs pH for the methionine resonance and it can be seen that the midpoint of the transition occurs at about pH 10.2.

The histidine C-2 peak is also present at pH values greater than 9.5. In the transition region there are two peaks present as shown in Figure 5.9 which consists of spectra, obtained at 100 MHz, of the aromatic region of α-chymotrypsin. The chemical shift scale is given in units of Hz from the solvent HOD peak and the two peaks occur at 290 and 296 Hz. This doublet is more clearly evident in the 220 MHz spectrum at pH 10.1 shown in Figure 5.10, where the two peaks occur at 625 and 638 Hz.

The methionine peak is not visible in spectra of trypsin over the pH range studied if accumulation of spectra is kept under three hours. If extensive autolysis

Figure 5.8


Methyl
∝-chymotrypsin
(a)
Methionine
4
3
2
1
0
δ

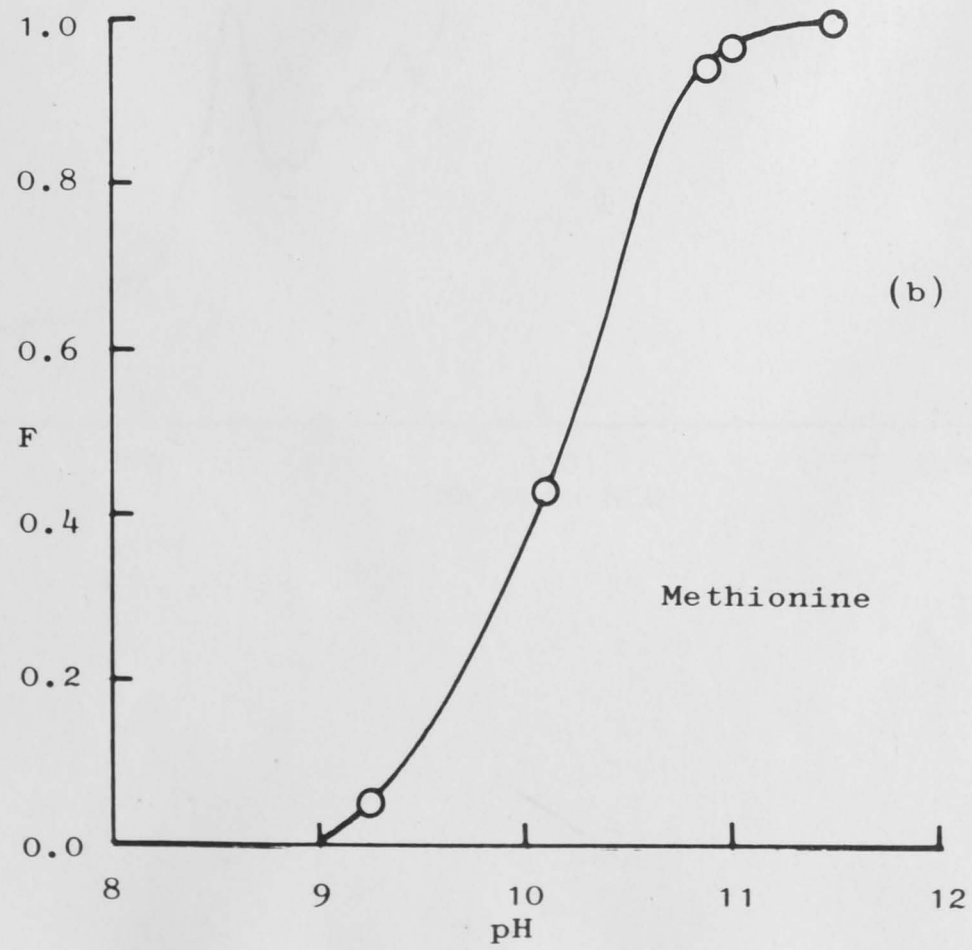

1.0
0.8
0.6
F
0.4
0.2
0.0
(b)
Methionine
8
9
10
11
12
pH

Figure 5.9

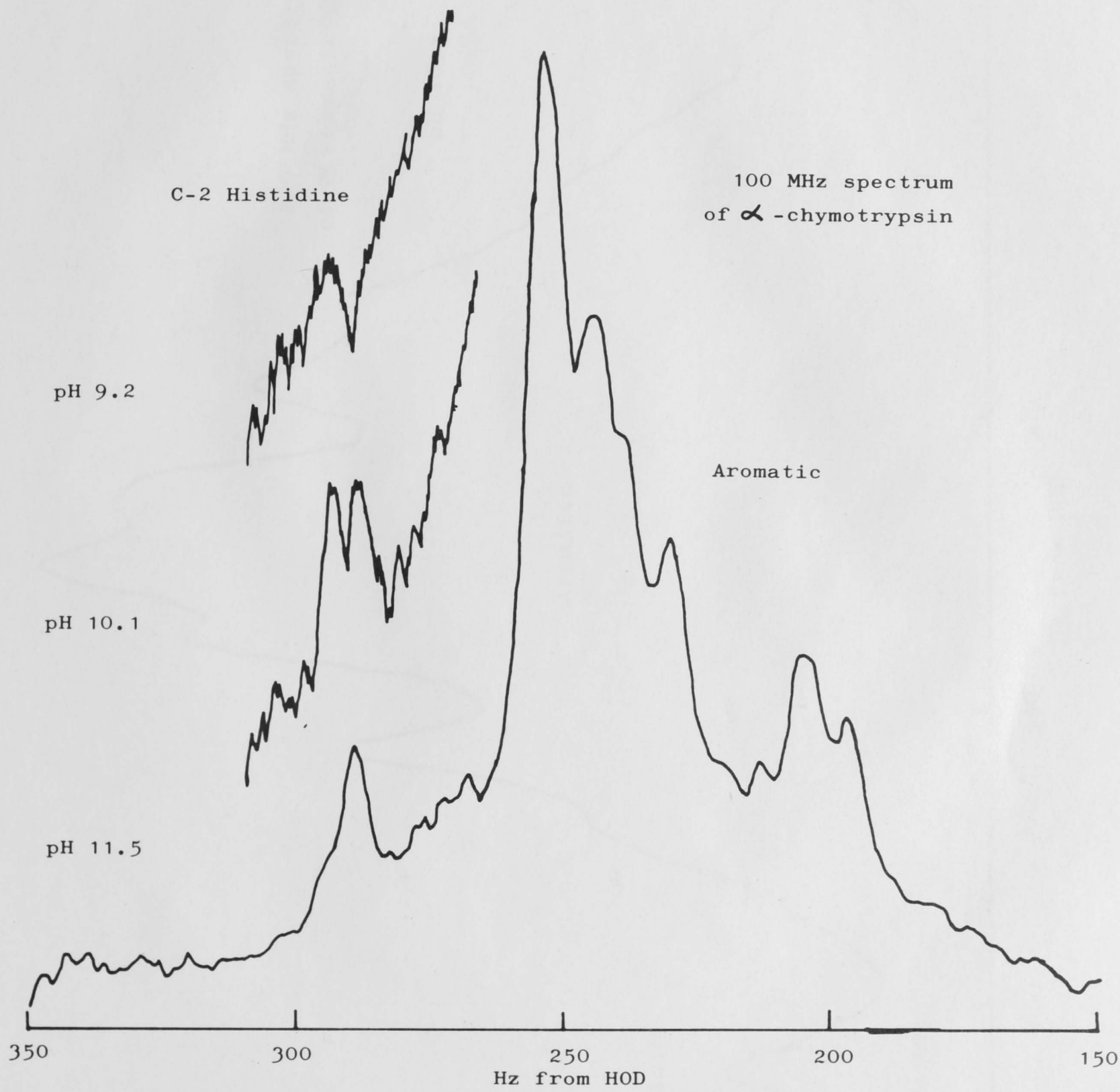

C-2 Histidine
100 MHz spectrum
of ∝ -chymotrypsin
pH 9.2
Aromatic
pH 10.1
pH 11.5
350
300
250
200
150
Hz from HOD

Figure 5.10

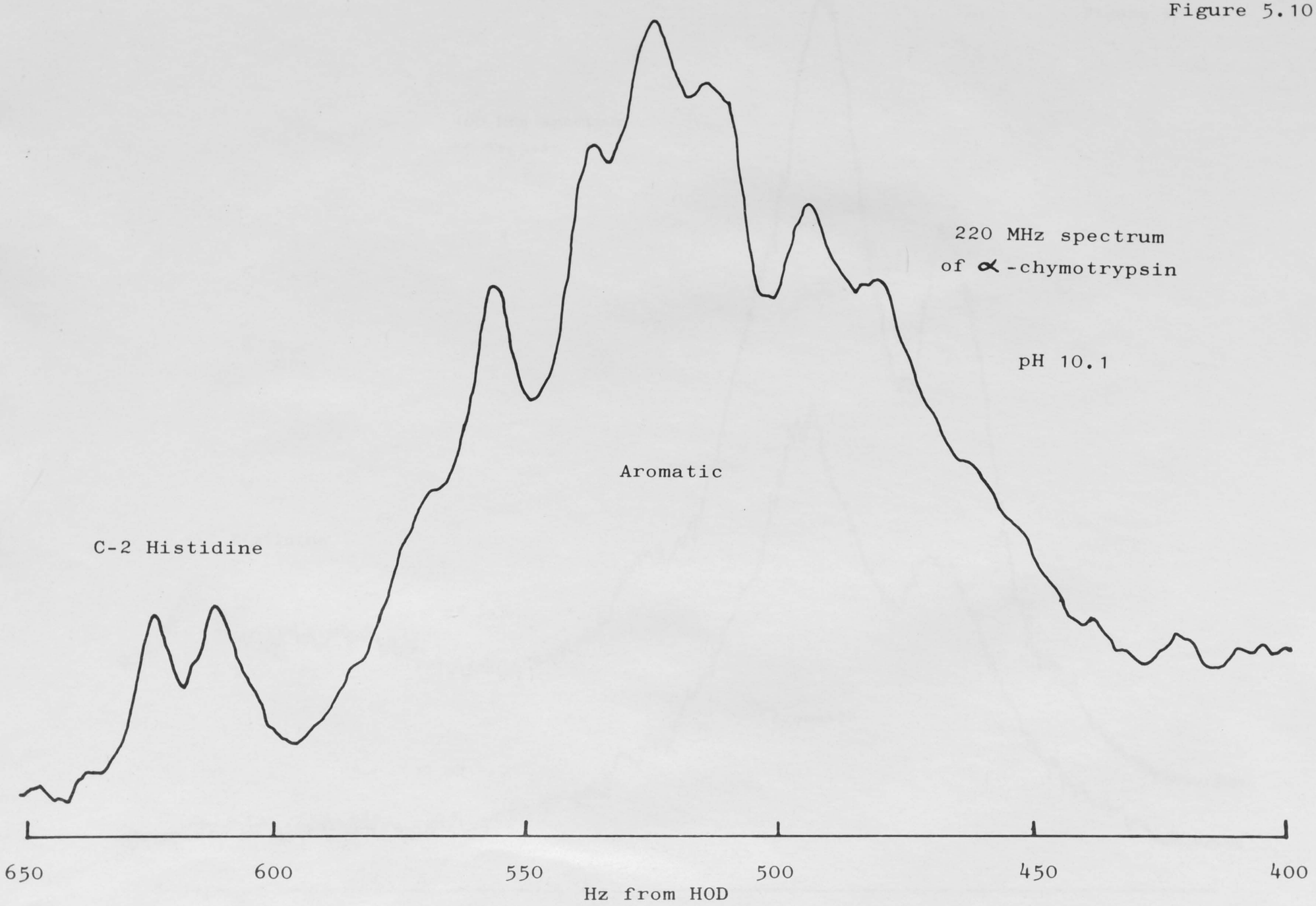
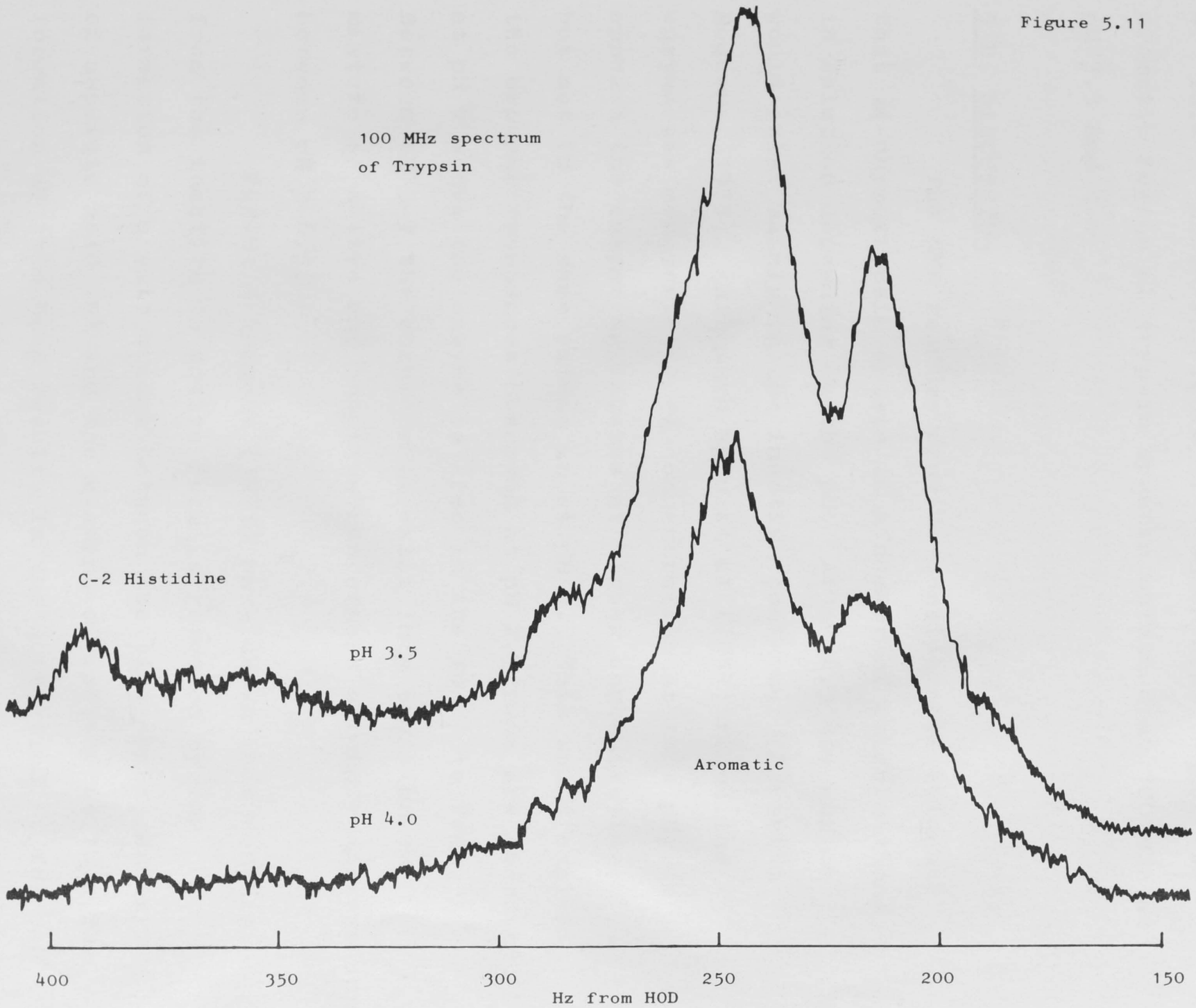

220 MHz spectrum
of ∝-chymotrypsin
pH 10.1
Aromatic
C-2 Histidine
650
600
550
500
450
400
Hz from HOD

Figure 5.11

100 MHz spectrum
of Trypsin



takes place the methionine peak is visible in the spectra. A histidine peak equivalent in area to one proton is present at pH values below 3.5 but not at pH values above 4. This is shown in Figure 5.11 which consists of the aromatic region of trypsin spectra obtained at 100 MHz at pH 3.5 and 4.0

5 D DISCUSSION

The NMR results provide considerable evidence that α-chymotrypsin exists in a number of conformations in solution depending on the pH. At pH 2.5 the enzyme would exist mainly in the inactive conformer (Fersht & Requena, 1971). Although Egan et al (1957) found the enzyme was monomeric at 2% concentration at this pH, it appears the enzyme aggregates at higher concentrations but not to the same extent as at pH 4. This would explain the broader resonances observed at pH 2.5 than are observed at pH 9 where the enzyme is also in the inactive form. Between pH 3-9 the enzyme will exist in a equilibrium mixture of active and inactive conformers strongly aggregating between pH 3-6.5.

Fersht & Requena (1971) have shown that conversion from the inactive to active form is governed by the formation of a salt bridge between the side chain carboxyl of aspartic acid 194 and the α-amino of isoleucine 16. The formation of this bond results in the activation of the binding site. The breaking of this salt bridge can be accomplished either by protonation of the aspartic carboxyl

or deprotonation of the isoleucine amino group. The authors were able to determine the apparent pK of the isoleucine amino group as $>$ 10 in the active conformer and 7.95 in the inactive conformer from the decending arm of the bell shaped curve of % active form vs pH. They were not able to determine a pK for the aspartic carboxyl group as the shape of the rising arm of the curve was not sigmoidal being distorted by the aggregation reaction. No significant differences could be seen in the 60 MHz NMR spectra of the monomeric active and inactive conformers so it seems reasonable to assume that their structures do not differ greatly. Although the peak widths of the inactive conformer are very small, it is not a completely denatured form of the enzyme as there is no sign of a histidine or methionine peak in the spectra.

Above pH 11 the enzyme exists in a denatured state as there is a further decrease in the width of the aromatic and methyl peaks and the methionine and histidine resonances are present in the spectrum at this pH. The midpoint of the F curve for the methionine peak occurs at pH 10.2 so that a group or groups with an apparent pK of 10.2 controls the denaturation reaction. The deprotonation of lysine side chain amino groups occurs at around this pH (Perrin, 1965).

A double histidine peak is present in the transition region. As no histidine peak is present in spectra of the inactive conformer, the double peak indicates that an intermediate configuration is present in the transition

region. The upfield peak appears to grow at the expense of the downfield peak. However, there are insufficient spectra to fully characterise this intermediate configuration. If the sample is left for three hours at pH 11.5 the denaturation is irreversible. Unfortunatly experiments were not carried out for shorter periods of time to observe if the denaturation was reversible.

Between pH 3-6.5 peak widths of α-chymotrypsin are very broad due to the aggregation reaction that is known to occur over this pH range. The fall in peak width at acid pH in spectra of 3-N-methyl-histidine 57-α-chymotrypsin implicates histidine 57 in the aggregation reaction. Several workers, Biltoren et al (1965), Sarfare et al (1966) and Johnson et al (1966), have stated that the active site is not involved in the aggregation reaction as they found aromatic inhibitors could bind to the polymeric species. However the binding site may not necessarily be obscured by aggregation involving the active site, and this would allow the binding of inhibitors to the polymeric species.

Aune & Timasheff (1971) studied the dimerization of α-chymotrypsin by sedimentation equilibrium over the pH range 2.3-5.5. The pH dependence of the equilibrium constant was accounted for in terms of short range electrostatic interactions between two pairs of identical ionizable groups in the enzyme, with apparent pK's of 5.0 and 3.6 in the monomer and 6.2 and 2.4 in the dimer. They identified the group with pK 5.0 and 6.2 as the imidazole group of histidine 57, and the group with pK 3.6 and 2.4

as the α-carboxyl group of tyrosine 146. Evidence to support this assignment has come from a number of workers. Gladner & Neurath (1954) found that the removal of tyrosine 146 by carboxypeptidase stopped aggregation and Neet & Brydon (1970) have shown that δ-chymotrypsin, in which tyrosine 146 is in a peptide linkage, does not dimerise. Egan et al (1957) found that photoxidation of histidine 57 stopped aggregation. A salt bridge between histidine 57 and tyrosine 146 has been shown to occur in the crystal structure of the dimer by Wright et al (1968).

The dependence of peak width on pH for DFP-α-chymotrypsin and FPS-α-chymotrypsin suggests that the introduction of a small group at serine 195, such as DFP, does not perturb the imidazole ring of histidine 57 sufficiently to prevent aggregation, while the presence of a large group, such as FPS, affects the imidazole ring sufficiently to prevent aggregation. These results agree with the work of Neet & Brydon (1970) who found that the presence of large groups at the active site prevented aggregation at pH 4 but not at pH 6, although there is still some aggregation present at pH 4 in the NMR experiments because of the high concentration of enzyme used.

Modification of histidine 57 is extremely efficient in preventing the formation of the salt bridge and stopping the formation of dimers at pH 4. It also stops the formation of trimers at pH 6 although this does not agree with the work of Neet & Brydon (1970) who found that modification of the histidine did not stop aggregation at this pH.

Trypsin shows very little change in peak width with pH over the range studied indicating that it does not aggregate in 0.01 M $CaCl_2$ solutions at pH 2.5-8. This is in agreement with the results of Cunningham et al (1953). Experiments could not be carried out at higher pH because of the precipitation of the enzyme.

The histidine C-2 resonance is absent in the 60, 100 and 220 MHz spectra of ∝-chymotrypsin up to pH 9. In trypsin one of the three histidines in the molecule gives rise to an observable resonance below pH 3.5. The two histidine residues in ∝-chymotrypsin, histidine 40 and 57, are both in the active site of the enzyme, while in trypsin two equivalent histidine residues, histidine 29 and 46, are in the active site while the other, histidine 79, is in another part of the molecule. As both enzymes have a similar amino acid sequence and three dimensional structure around the active site (Dayhoff & Eck, 1968; Hartley, B. S., 1970), one can reasonably assume that the histidine C-2 resonance observed in trypsin at pH 3.5 is from histidine 79, outside the active site. Its disappearance could be due to a slight conformational change in the molecule controlled by a group ionizing at an apparent pK 3.5-4. This would implicate a carboxyl group. As Hartley (1970) has only shown that the trypsin sequence can be fitted to the X-ray model of ∝-chymotrypsin, the true coordinates of the atoms of trypsin are not known and thus the distance between histidine 79 and any nearby carboxyl groups can not be calculated.

The absence of a C-2 histidine resonance in α-chymotrypsin, between pH 3-6.5, could be due to broadening caused by aggregation especially for histidine 57 which is involved in a salt bridge. Above pH 7 the enzyme exists as a monomer and broadening can not be caused by aggregation. There is little chance of exchange broadening of the histidine resonance between active and inactive forms of the enzyme, because if there was exchange between two different environments it would have to occur at a rate $\approx 2\pi(V_a - V_i)$, where V_a and V_i are the chemical shifts of histidine resonances in the active and inactive conformers, to cause any significant broadening. Since Fersht & Requena have shown that exchange between active and inactive species is slow and $(V_a - V_i)$ increases by a factor of 3.7 from 60-220 MHz this is not the case.

The other effect that could result in broadening of the histidine resonances is dipole-dipole interaction. King (1970) has calculated from the coordinates of tosyl-α-chymotrypsin, supplied by Dr. J. J. Birktoft, that the C-2 proton of histidine 57 comes within 2.0 Å of one of the CB protons of serine 195. The C-2 proton of histidine 40 is within 2.6 Å of the CA proton of serine 32 and 2.9 Å from the CE3 proton of tryptophan 141.

When the above values of $\underline{b}$ are substituted into the equation describing dipole-dipole broadening (Chapter 2), a value of 12 Hz for the C-2 proton of histidine 57 and 4 Hz for the C-2 proton of histidine 40 is obtained for the dipole-dipole contribution to the peak width. This would

give a total width of 15 Hz for the C-2 resonance of histidine 57 and 7 Hz for histidine 40 as the width of the peak in the free amino acid is 3 Hz. There is also the possibility of further broadening if the protons attached to imidazole nitrogens do not exchange with deuterium or if the protons of a water molecule close to histidine 40 fail to exchange with deuterium. Having regard to the errors inherent in the calculations, it seems likely that the C-2 resonances in ∝-chymotrypsin are too broad to observe (width > 15 Hz) because of the close proximity of protons from other residues, which would play a dominant part in the relaxation mechanism. A similar effect may be the cause of the broadening of the peaks from the two equivalent histidine residues in trypsin. It should be possible, in principle, to use the equation describing dipolar broadening in reverse and calculate interproton distances from the measured values of peak width.

In native proteins the $-SCH_3$ methionine resonances are almost always not observed (Bradbury, Chapman & King, 1971). There are two reasons for this. Firstly, the resonance occurs at 2.14 δ, on the shoulder of a very large broad resonance and it can not be seen unless it is reasonably sharp (< 10Hz). Secondly, each proton in the $-SCH_3$ group is relaxed by dipole-dipole interactions with the two other protons which are closely placed (b=1.8 Å). If the two methionine residues are held rigidly and their rotational motion is the same as that of ∝-chymotrypsin as a whole, calculations give a dipole-dipole contribution

to the width of 42 Hz. This width is much larger than could possibly be observed and accounts for the absence of the $-SCH_3$ peak in α-chymotrypsin. Trypsin, with a molecular weight and shape similar to that of α-chymotrypsin, would also be expected to have peak widths of the same order if the methionine groups are held rigidly.

CHAPTER 6

THE DENATURATION OF TRYPSIN, ∝-CHYMOTRYPSIN AND S-CH_3-METHIONINE 29-RIBONUCLEASE-A.

6 A INTRODUCTION

Harris (1956) studied the effect of urea on the conformation of trypsin and ∝-chymotrypsin. Viscosity and enzymic activity measurements were used to follow the extent of denaturation at a number of urea concentrations. For trypsin, viscosity measurements were made at pH 4.0 and enzymic activity measurements at pH 7.8 in separate experiments. Both sets of measurements gave smooth sigmoidal denaturation curves, with mid-points at 4 M urea in each case. When the solutions used in viscosity studies were diluted at pH 7.8, full enzymic activity was recovered indicating that denaturation was reversible at pH 4.0. Harris (1956) analysed the results in terms of a rapid equilibrium between the native enzyme and an unfolded form.

Viscosity and enzymic activity measurements, for ∝-chymotrypsin, were made at pH 5.0 and 7.8 respectively. Both sets of measurements gave smooth sigmoidal denaturation curves with mid-points at 5.5 M urea. When solutions used in viscosity studies were diluted at pH 7.8 it was found that the activity of the recovered enzyme fell with increasing urea concentration in the original solutions used for viscosity studies. Harris (1956) concluded that denaturation by urea at pH 5.0 was irreversible. The results were analysed in terms of a single step transition between the

native enzyme and an irreversibly denatured form.

The results of Harris (1956) for the denaturation of trypsin by urea were confirmed by Riordan et al (1961) who measured the sedimentation constants of trypsin in urea solutions at pH 5.0. The results obtained were consistent with an increase in molecular asymmetry with increasing urea concentrations. The transition between the native and denatured forms of the enzyme occurred between 3-5 M urea.

Martin (1962) studied the rate of denaturation of α-chymotrypsin by 8 M urea at a number of pH values. He found that the rate of inhibition of enzymic activity was relatively fast (60 seconds at pH 6.2) but the rate of unfolding of the enzyme was slow, taking one hour to reach equilibrium at pH 6.2. He concluded that inhibition of activity is not necessarily caused by the unfolding of the enzyme and may be due to interactions between urea and certain residues in the active site. Bradbury and King (1972) studied an analogous reaction in ribonuclease and found that small concentrations of urea inactivated the enzyme by binding to the active site histidine residues 12 and 119.

Most of the work in this field, since the publication of Martin (1962), has been concentrated on measuring the rates of denaturation of α-chymotrypsin by high concentrations of urea (6-8 M) under various conditions. Chervenka (1962) and Hopkins & Spikes (1967) found that the rate of denaturation of α-chymotrypsin was very slow at pH 7, taking up to three hours to reach

equilibrium at low urea concentrations. The rate of denaturation increases by a factor of 10-15 at acid and alkali pH.

Hopkins & Spikes (1968) reported that the pH-rate of denaturation profile for trypsin in 8 M urea was similar to that of ∝-chymotrypsin with maximum stability at pH 7. The denaturation by urea of both trypsin and ∝-chymotrypsin is a first order reaction, governed by the rate of unfolding of the native enzymes. Solutions of the enzymes in urea are regarded as consisting of native and unfolded forms undergoing slow exchange at neutral pH and faster exchange at acid and alkali pH.

The denatured forms could be random coils within the limits imposed by disulphide bridges. However Bradbury & King (1969) noticed the presence of a double methionine resonance in NMR spectra of trypsin in 8 M urea and 6 M guanidine.HCl at pH 4.7 and 8.0, indicating the presence of noncovalent interactions in the denatured conformer. The methionine assignment was confirmed by methylation with methyl iodide which resulted in the disappearance of the double methionine peak and the appearance of a new peak at the correct position for the methyl peak of S-methyl methionine. The spectra of ∝-chymotrypsin in 8 M urea showed no anomalies and the conformer appears to be a random coil.

A double methionine peak has also been observed during the denaturation of ribonuclease-A by formic acid (Bradbury & King, 1972). The additional methionine peak is upfield from the normal methionine position. This effect

is caused by shielding of the $-SCH_3$ protons of a methionine residue due to either the interaction of the methyl group with the face of an aromatic ring or the presence of a nearby negatively charged group.

This chapter will be concerned with the denaturation of α-chymotrypsin by urea, trypsin by urea and guanidine.HCl and S-methyl-methionine 29-ribonuclease-A by formic acid, paying particular attention to the methionine resonances.

6 B EXPERIMENTAL

For experiments involving the unfolding of trypsin and α-chymotrypsin by urea, a 10% solution of the enzyme under study was adjusted to the required pH and a weighed amount of urea was added, the pH being readjusted if necessary. Solutions were left standing for one hour before spectrum accumulation was begun. This procedure was adopted to allow equilibration to take place between the native and denatured forms of the enzyme. After this period of time the spectrum was accumulated for approximately 4 hours (130 scans) on the 60 MHz spectrometer. It was noticed that solutions containing less than 4 M urea formed gels after approximately sixteen hours. Broadening of peaks occurred after about eight hours and spectrum accumulation could not be carried out for more than six hours without the possibility of this broadening affecting the accumulated spectrum.

A similar procedure was followed in experiments involving the unfolding of trypsin by guanidine.HCl.

Molal urea and guanidine.HCl concentrations were converted to molar units according to Kawahara & Tanford (1966). The addition of these denaturants causes an expansion in the volume of the solution. Peak heights were corrected for the dilution accompanying increases in denaturant concentration.

In the unfolding experiments involving S-methyl-methionine 29-ribonuclease A, small volumes of d_2-formic acid were successively added, by means of micropipetts, to a solution of the enzyme initially at 15% (w/v) concentration at pH 4 in 0.5 ml. D_2O. This addition of formic acid was continued until the acid concentration was 15% by volume. Between the additions of acid the spectrum was accumulated for three hours on the 60 MHz spectrometer. For higher acid concentrations, protein solutions were prepared by mixing appropriate volumes of acid and D_2O.

The concentration of acid was measured by a calibration curve of chemical shift of the HOD peak vs formic acid concentration (Bradbury & King, 1972).

6 C RESULTS

Spectra of α-chymotrypsin in D_2O and 8 M urea at pH 3 are shown in Figure 6.1. Sharpening of peaks, especially the methionine and methyl peaks, is evident in the spectrum of the denatured protein. The resonance from histidine C-2 protons is not visible due to the noise level in the baseline caused by the short accumulation time.

The extent of unfolding, F, was calculated for the

Figure 6.1

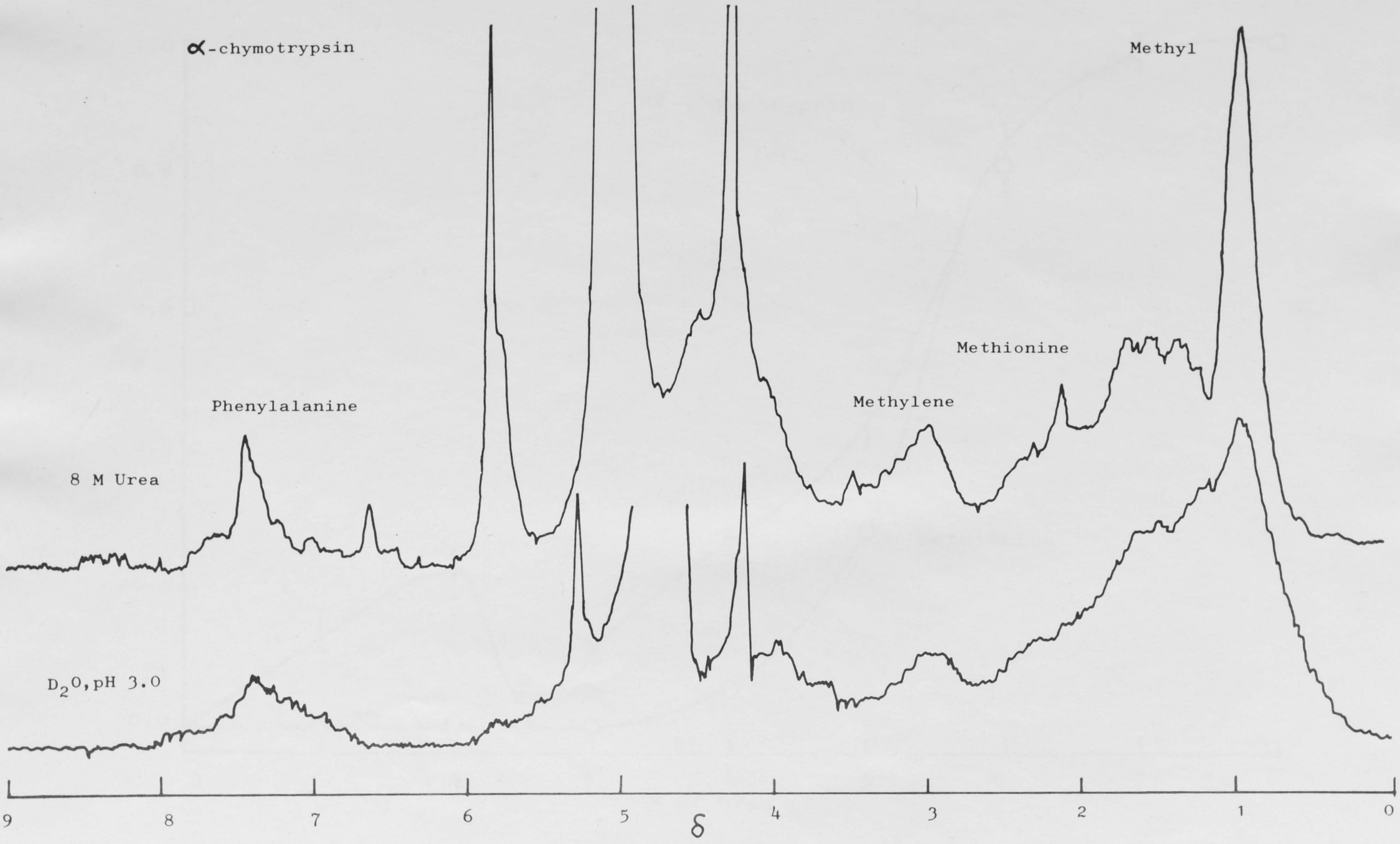
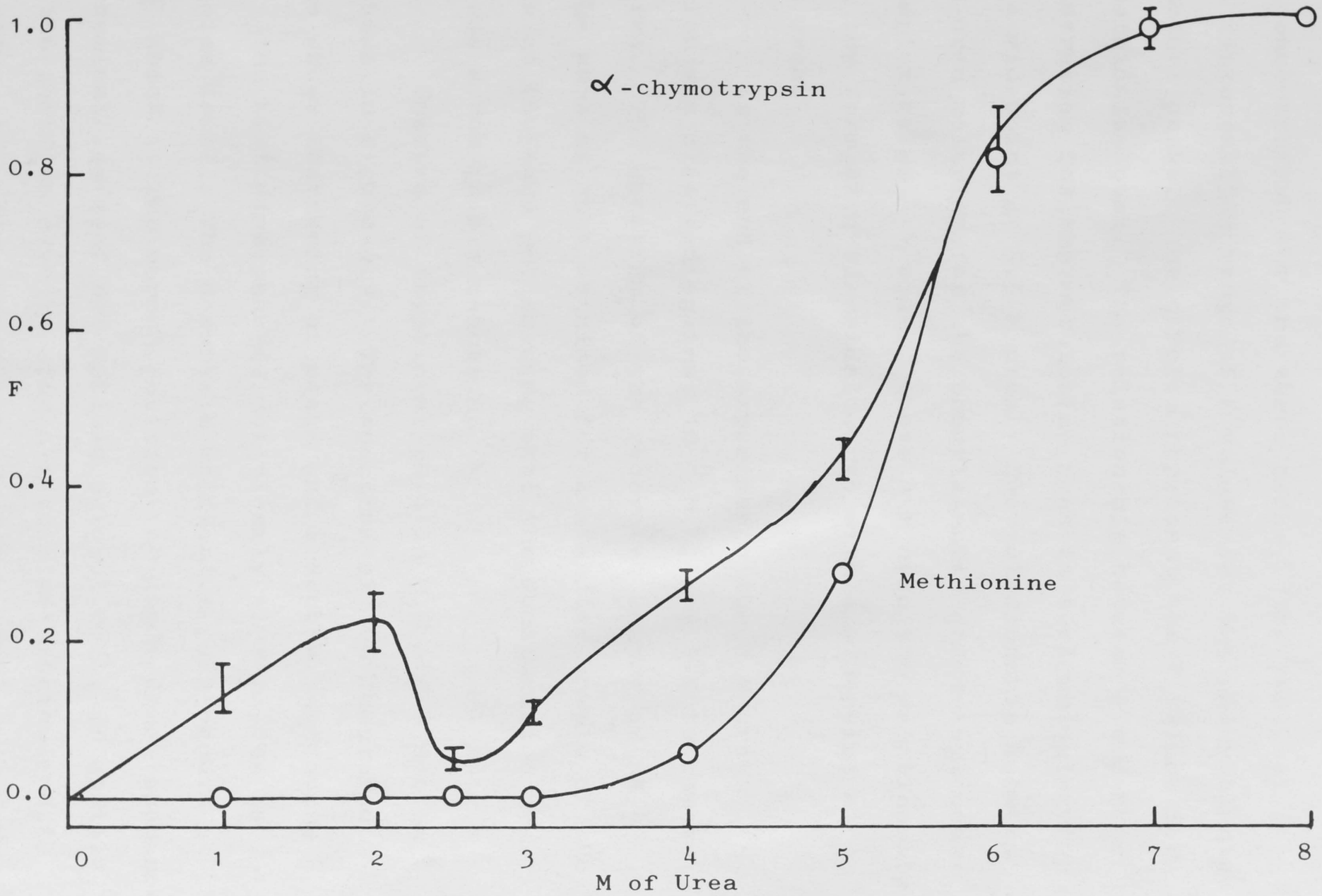

α-chymotrypsin
Methyl
Methionine
Methylene
Phenylalanine
8 M Urea
D_2O, pH 3.0
9
8
7
6
5
δ
4
3
2
1
0

Figure 6.2


α-chymotrypsin
Methionine
1.0
0.8
0.6
F
0.4
0.2
0.0
0
1
2
3
4
5
6
7
8
M of Urea

phenylalanine, methyl and methionine peaks. At urea concentrations less than 4 M, the methionine peak was not visible. Figure 6.2 shows the relationship between F and urea concentration for the three peaks. The vertical lines represent the range of F values for the phenylalanine and methyl peaks. The circles represent the F values for the methionine peak. The relationship between F and urea concentration for the methionine peak is a sigmoidal curve with a mid-point at 5.5 M urea. The relationship between F and urea molarity for the phenylalanine and methyl peaks is complex below 4 M urea. Above 4 M urea the relationship is in the form of a sigmoidal curve with a mid-point at 5.2 M urea.

At the end of the experiments the 8 M urea solution was dialysed against 10^{-3} M HCl at 4^{o}C to remove the urea. The spectrum of the recovered enzyme at pH 4 was the same as that obtained for a standard sample of the enzyme at the same pH, showing that the denaturation by urea was a reversible reaction.

Spectra of trypsin at pH 4 in D_2O and 8 M urea are shown in Figure 6.3. The spectrum of the denatured enzyme shows sharpening of peaks and a double methionine peak. The histidine peak is not clearly visible due to the noise level. The downfield methionine peak occurs at 2.15 δ which is the normal position for methionine resonances. The chemical shift of the upfield peak, 2.08 δ, is upfield from the position expected for a normal methionine and is indicative of a noncovalent interaction between the methionine

methyl group and the face of a aromatic ring or an interaction with a negatively charged group. As the area of the two peaks is the same and trypsin contains two methionine residues, the simplest explanation of this effect is that one of the methionine residues is in a normal environment while the other is in a perturbed environment. The F values of the phenylalanine, tyrosine, methylene, methyl and downfield methionine peaks were measured as a function of urea concentration. The range of F values is plotted in Figure 6.4 as a function of urea concentration. The F curve is sigmoidal with a mid-point at 4.2 M urea. The shape of the curve is indicative of a single step transition between native and unfolded forms of the enzyme.

The 8 M urea sample was dialysed against 10^{-4} M HCl at 4^{o}C to remove the urea. The spectrum of the recovered protein at pH 4 was the same as a reference spectrum at the same pH, indicating that the denaturation was reversible.

Figure 6.5 (a) shows the upfield region of the spectrum of trypsin in guanidine.HCl (6 M). The upfield region of the spectra of trypsin in 8 M urea and 6 M guanidine.HCl were identical. The chemical shifts and shapes of the double methionine resonances were the same in both cases. These observations indicate that the conformation of the unfolded form is the same in 8 M urea and 6 M guanidine.HCl. Figure 6.5 (b) shows the transition curve of trypsin in guanidine.HCl at pH 4. The vertical lines, in the transition curve, represent the range of F values for the methylene, downfield methionine and methyl peaks.

Figure 6.3

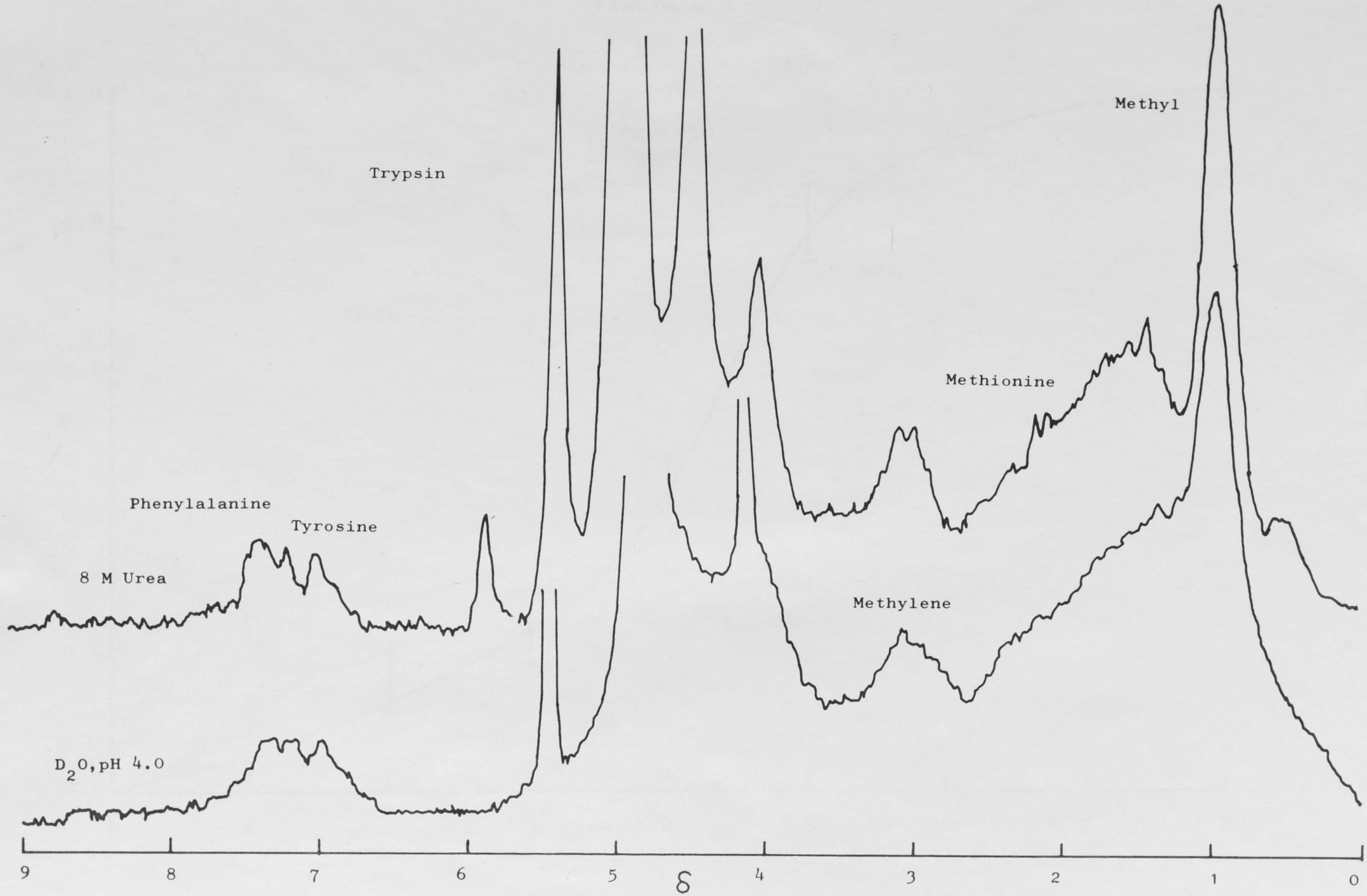

Trypsin
Methyl
Methionine
Phenylalanine
Tyrosine
8 M Urea
Methylene
D_2O, pH 4.0
9
8
7
6
5
δ
4
3
2
1
0

Figure 6.4

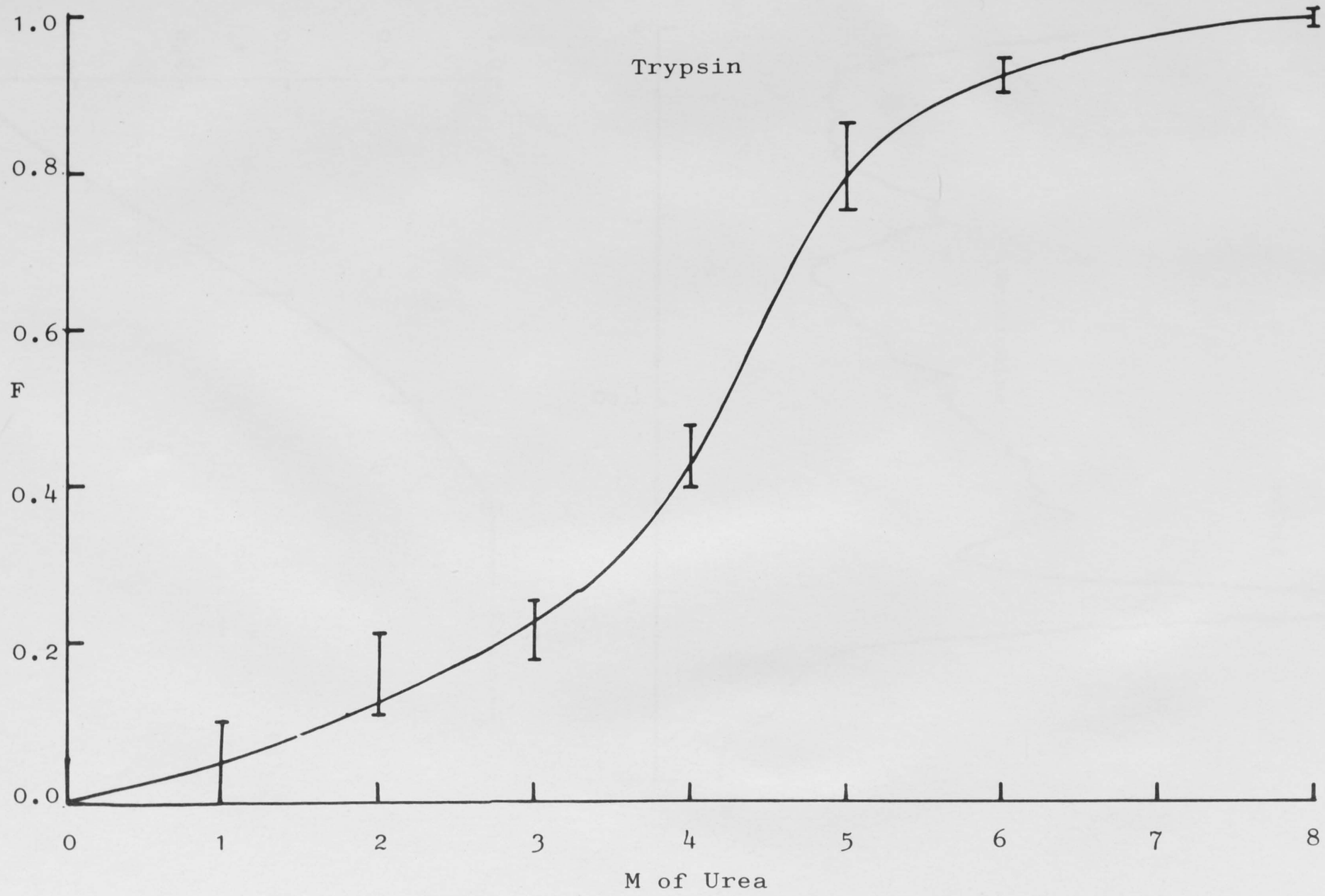
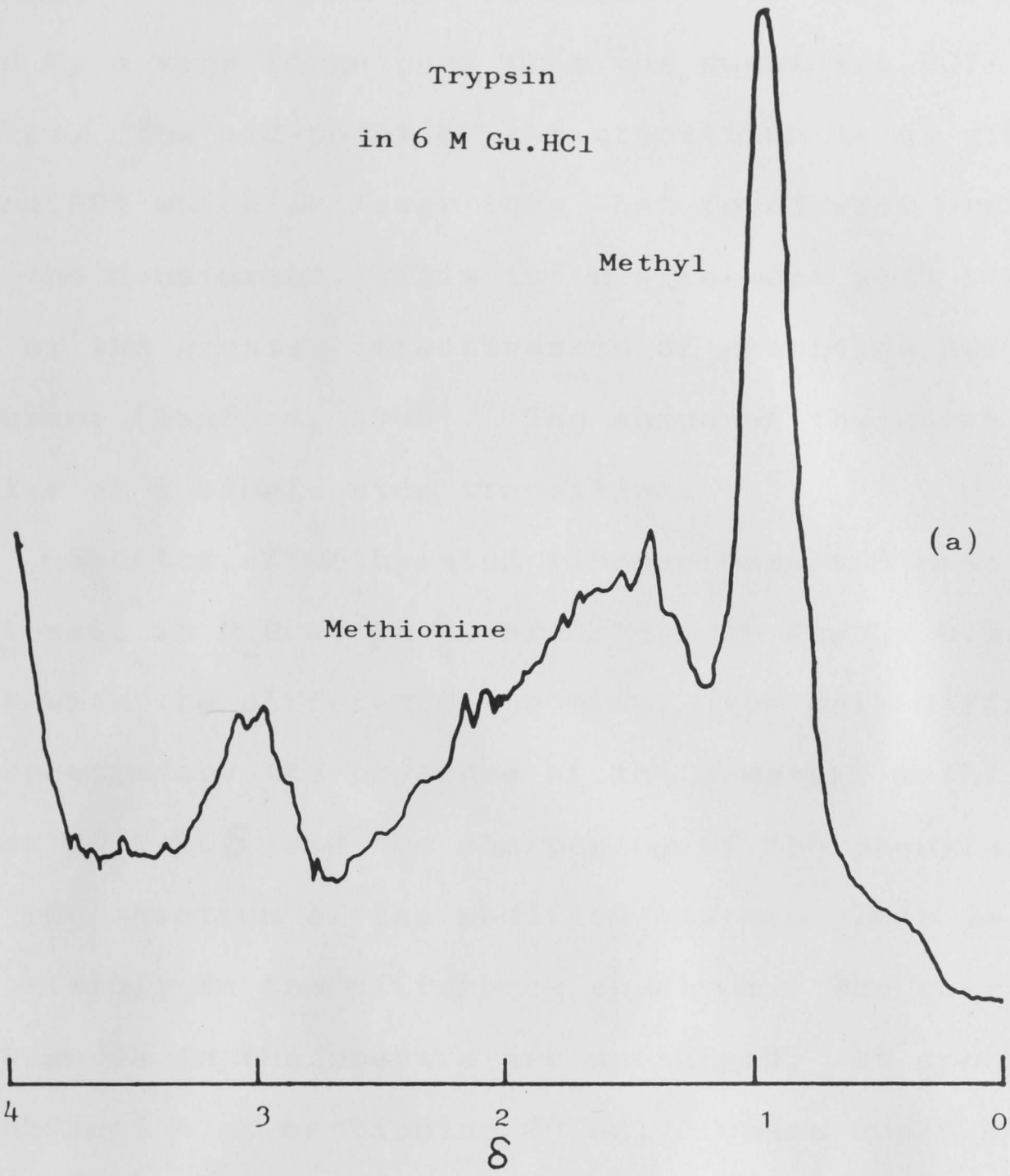
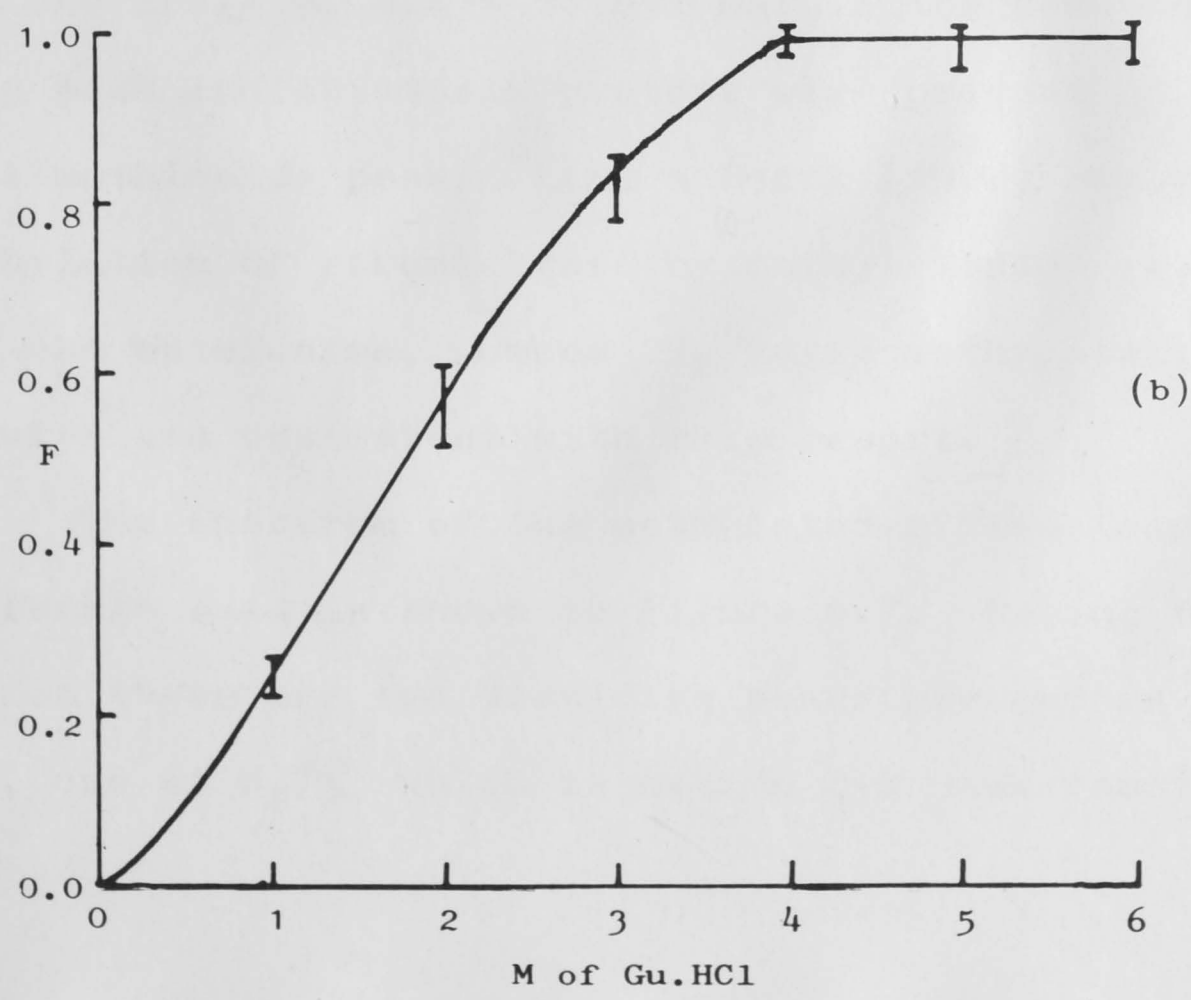


Figure 6.5


Trypsin
in 6 M Gu.HCl
Methyl
Methionine
(a)
4
3
2
1
0
δ


1.0
0.8
0.6
F
0.4
0.2
0.0
0
1
2
3
4
5
6
M of Gu.HCl
(b)

The aromatic peaks could not be measured as they were obscured by a very large peak from the guanidine.HCl resonances. The mid-point of the transition is at 2.0 M guanidine.HCl which is lower than that found when urea is used as the denaturant. This is in agreement with other reports of the greater effectiveness of guanidine.HCl as a denaturant (Tanford, 1968). The shape of the curve is indicative of a single step transition.

Spectra of methylated ribonuclease and native ribonuclease, in D_2O at pH 4, are shown in Figure 6.6, together with the difference spectrum. The only differences in the spectra are the presence of the S-methyl methionine resonance at 2.90δ and the sharpening of the phenylalanine peak in the spectrum of the modified enzyme. This is clearly visible in the difference spectrum. The rest of the resonances in the spectra are unchanged. It appears that methylation of methionine 29 only causes small changes in the conformation of the native enzyme. A comparison between the areas of the S-methyl methionine peak and the aromatic peak indicated six protons were present in the S-methyl methionine peak. Link & Stark (1968) reported that the methylation of ribonuclease by methyl iodide results in only one methionine, number 29, being methylated. The NMR results are consistent with this report.

The spectrum of the methylated ribonuclease in 10% d_2-formic acid is shown in Figure 6.7. During the transition there are two histidine peaks present in the spectra, one at 8.7δ which is native C-2 peak consisting

Figure 6.6

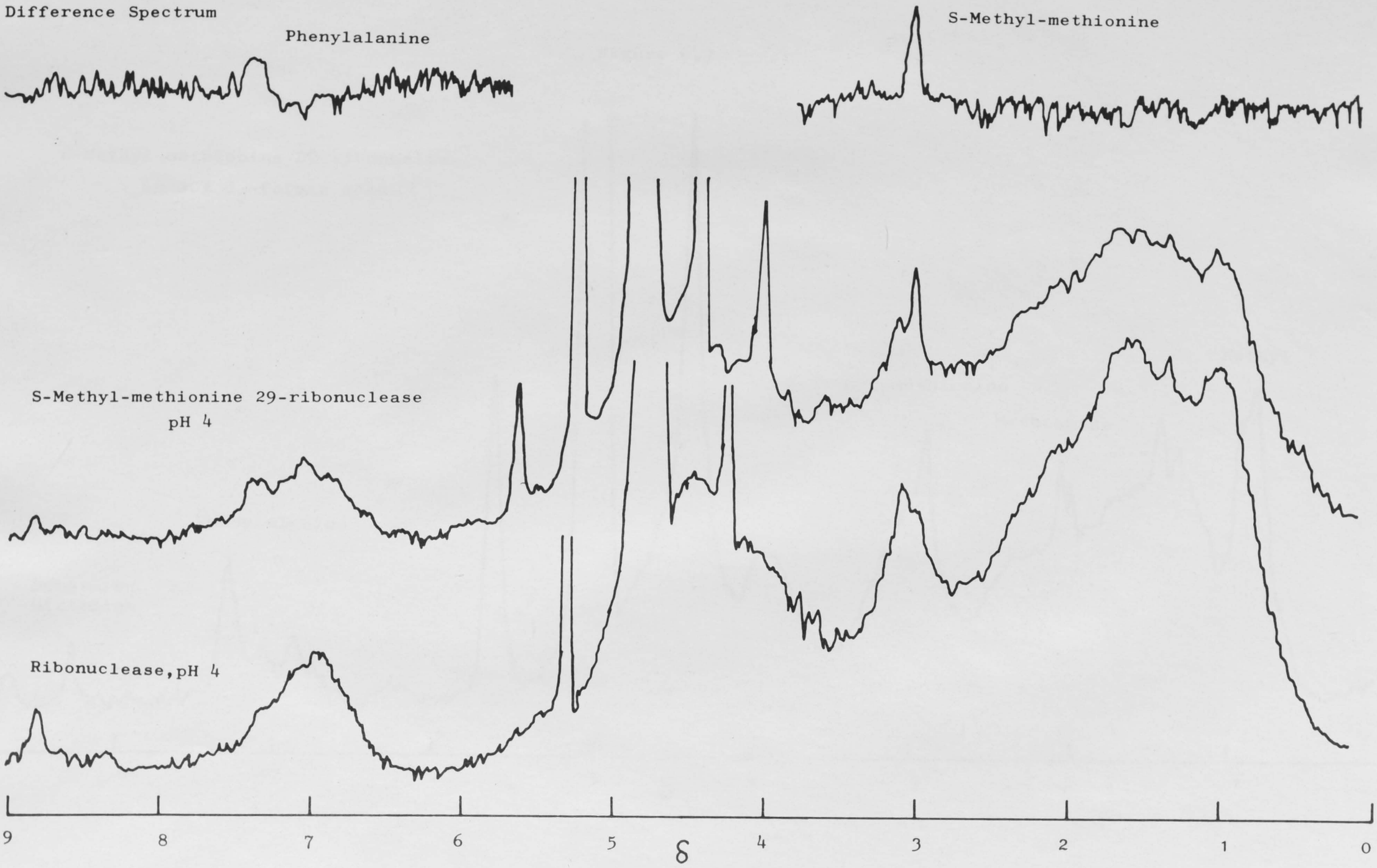
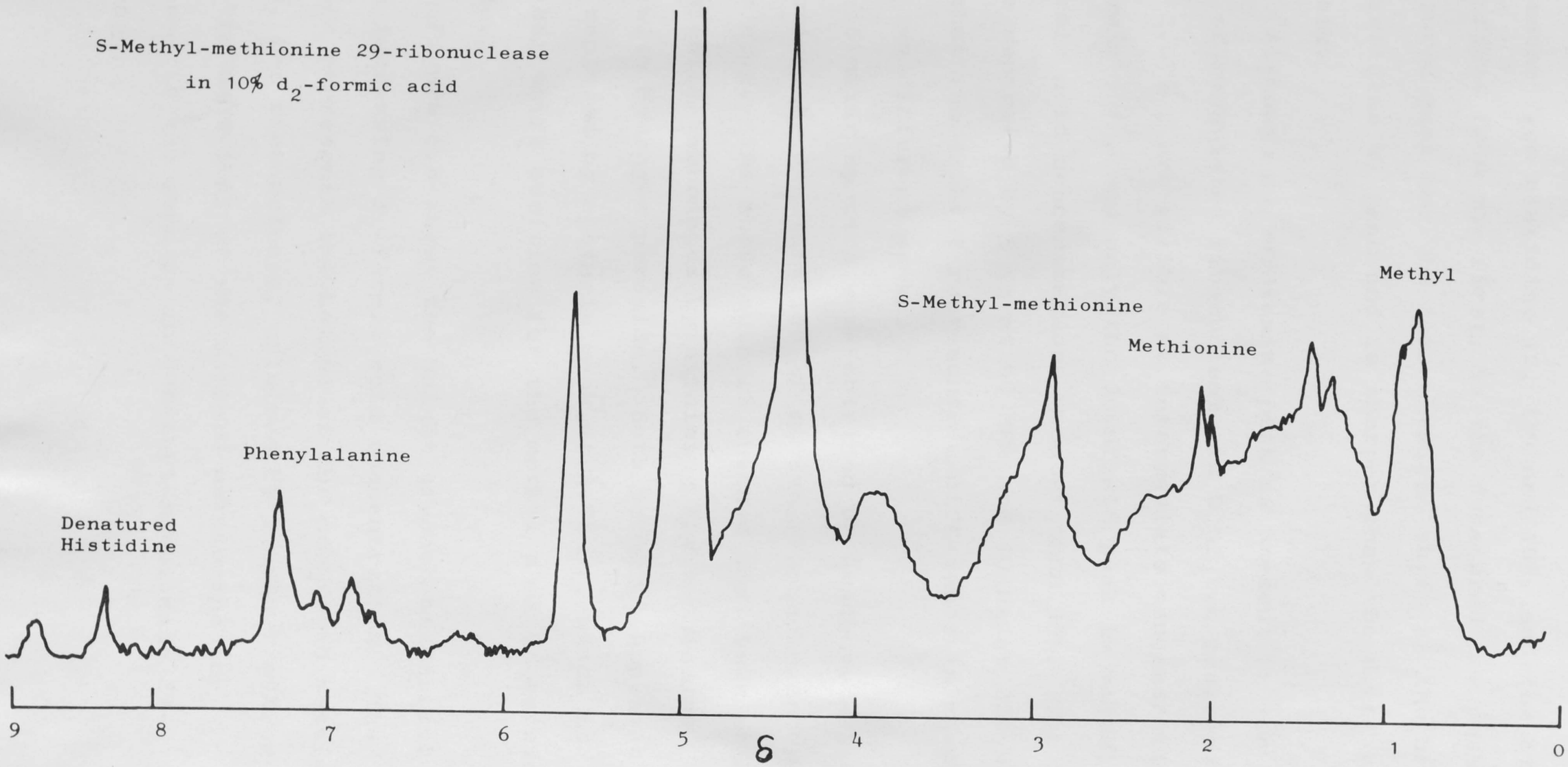

Difference Spectrum
Phenylalanine
S-Methyl-methionine
S-Methyl-methionine 29-ribonuclease
pH 4
Ribonuclease, pH 4
9
8
7
6
5
δ
4
3
2
1
0

Figure 6.7


S-Methyl-methionine 29-ribonuclease
in 10% d_2-formic acid
Methyl
S-Methyl-methionine
Methionine
Phenylalanine
Denatured
Histidine
9
8
7
6
5
δ
4
3
2
1
0

of resonances from histidine 12, 119 and 105, and the other, 0.15δ upfield from the first, is the denatured C-2 peak. The denatured peak has the same chemical shift as the broad native histidine 48 peak and is sharper than the downfield native peak.

Although no methionine peak is present in the spectrum of methylated ribonuclease in D_2O, two peaks, at 2.12 and 2.07δ, are visible at intermediate concentrations of d_2-formic acid, and only the downfield peak is visible at d_2-formic acid concentrations greater than 35%. The two peaks are separated by 3 Hz at 60 MHz and 10 Hz at 220 MHz, showing that the doublet represents a difference in chemical shift of two different peaks.

Similar spectra were obtained by Bradbury & King (1972) during the denaturation of native ribonuclease by d_2-formic acid. The above authors checked that both peaks were methionine resonances by oxidising the methionine residues with hydrogen peroxide. Both peaks disappeared and were replaced by a single peak at 2.78δ, which is the chemical shift position for the methyl peak of methionine sulphoxide.

Figure 6.8 shows the height of each methionine peak with increasing d_2-formic acid concentration. The dotted line represents the height of the downfield methionine resonance, for ribonuclease, obtained by Bradbury & King (1972). The behaviour of the upfield methionine in ribonuclease is the same as in S-methyl-methionine 29-ribonuclease.

Figure 6.8

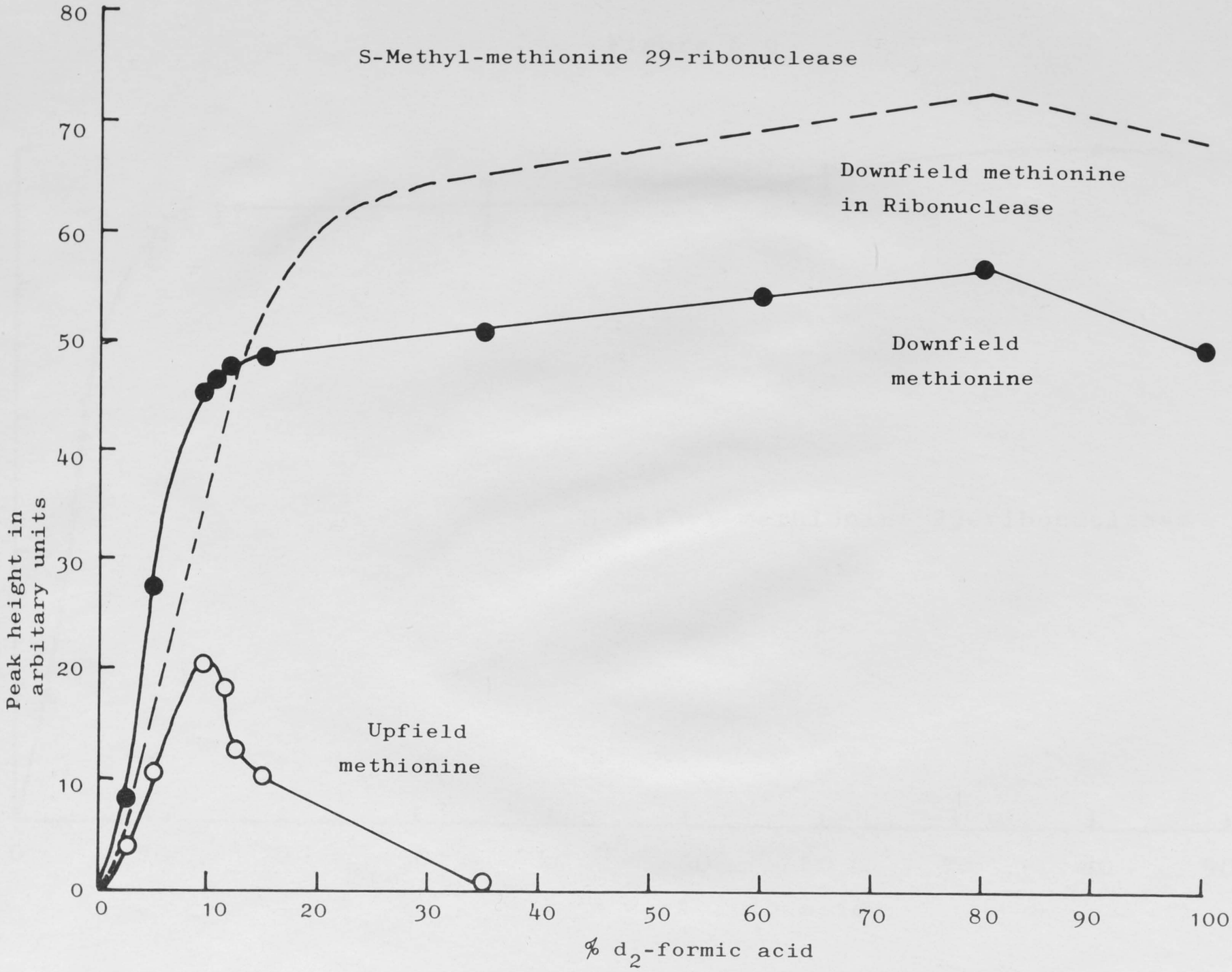
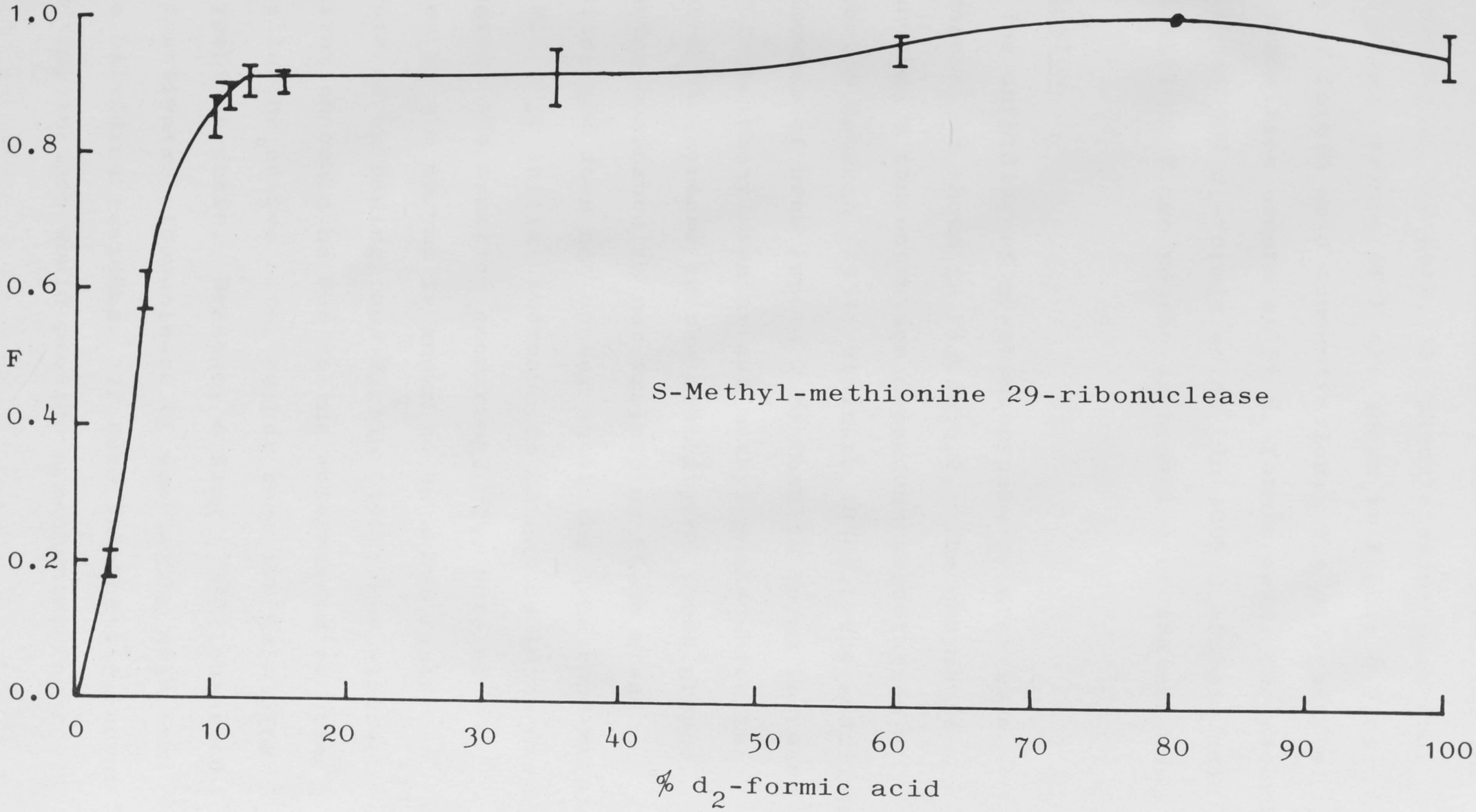

S-Methyl-methionine 29-ribonuclease
Downfield methionine in Ribonuclease
Downfield methionine
Upfield methionine
Peak height in arbitary units
% d_2-formic acid
0
10
20
30
40
50
60
70
80
90
100

Figure 6.9


1.0
0.8
0.6
F
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
S-Methyl-methionine 29-ribonuclease
% d2-formic acid

The extent of unfolding, F, was measured for the denatured histidine C-2 peak, the phenylalanine peak and the methyl peak. Values of F are shown in Figure 6.9 as a function of formic acid concentration. The mid-point of the first transition occurs at 5% d_2-formic acid, the second occurs at about 50% d_2-formic acid. In 100% d_2-formic acid there is a fall in F due to the aggregation of the enzyme.

6 D DISCUSSION

The unfolding of ∝-chymotrypsin by urea is a complex process, as shown in Figure 6.2. The enzyme is known to undergo a concentration dependent association at pH 3 (Schwert & Kaufman, 1951; Steiner, 1954). The addition of small amounts of urea (up to 2 M) results in an increase in height of the phenylalanine and methyl peaks which is indicative of an increase in the mobility of these groups. The methionine resonance is not visible at these urea concentrations and does not appear until the urea concentration reaches 4 M. This initial increase in height could be due to a disaggregation reaction occurring. The rate of inactivation of the enzyme is known to be a lot faster than the rate of unfolding, and Martin (1962) has stated that inactivation could be due to the interaction of urea with groups in the active site, rather than the unfolding of the polypeptide chain. Bradbury & King (1972) observed that urea inactivates ribonuclease by complexing with the active site histidine residues. If such interactions occur in ∝-chymotrypsin they would provide a mechanism for

disaggregation, as Aune & Timasheff (1971) have shown that histidine 57, in the active site, is involved in the aggregation reaction.

The height of the two peaks falls at 2.5 M urea and then starts to increase at higher urea concentrations. This appears to be due to a time dependent aggregation reaction occurring, although it is not clear why this should happen. It was noticed, during the unfolding experiments, that the greatest tendency for solutions to form gels occurred between 2 and 3 M urea concentrations. Gels formed very rapidly (within 4 hours) at these urea concentrations if the pH was adjusted to 4.0. This probably indicates that a carboxylate anion is involved in this time dependent aggregation reaction. Aune and Timasheff (1971) have shown that the α-carboxyl of tyrosine 146 is involved in the aggregation reaction that occurs in the absence of urea. This group forms a salt bridge with the cationic form of the imidazole ring in histidine 57. However it is unlikely that this reaction is the cause of the time dependent aggregation, as it is highly improbable that this salt bridge would be stabilised at 2-3 M urea concentrations and destabilised at lower concentrations. Some other form of bonding must be responsible for the time dependent aggregation reaction.

The methionine peak appears above 4 M urea and its F values merge with those of the other peaks at 6 M urea. This behaviour can be explained by assuming that the enzyme is monomeric at a concentration of 4 M urea, and the unfolding

reaction occurs above this concentration. The F value for the phenylalanine and methyl peaks is higher than the F value for methionine at 4 and 5 M urea because these peaks have become sharper due to the disaggregation reaction while the methionine peak is unaffected by disaggregation. This, together with sharpening due to unfolding, would give them a higher F value than that for the methionine peak.

The relationship between F and urea concentration for the methionine peak is in good agreement with the results obtained by Harris (1956) for the denaturation of α-chymotrypsin. The above author did not observe any anomalous behaviour at low urea concentrations. This could be due to the lower protein concentrations used, (1%), where there would be far less aggregation compared with the 10% solutions used for NMR studies.

A summary of the results indicates that urea concentrations below 4 M remove the original aggregation present in the enzyme without unfolding it. A secondary time dependent aggregation also takes place at these low urea concentrations. In the main transition region, above 4 M urea, the enzyme exists in a mixture of native and unfolded molecules in slow equilibrium (Chervenka, 1962; Hopkins & Spikes, 1967), with a mid-point in the transition curve at 5.5 M urea.

Although Harris (1956) reported that denaturation is irreversible at pH 5, it appears that it is reversible at pH 3. This discrepancy can be explained by the results obtained by Martin & Frazier (1963) who found that the pH

at which 8 M urea solutions of the enzyme are diluted must be below 3 to obtain the native enzyme. If the pH of the diluting solution is greater than 3, the denatured enzyme refolds to a non-native conformation that has no enzymic activity. One or more carboxylic acid groups must be protonated if the denatured enzyme is to refold to its native conformation. If denaturation is carried out at pH 3 the enzyme will exist in a mixture of native and unfolded conformers in the transition region. At pH 5, however, the enzyme would exist in a mixture of native, unfolded and refolded non-native conformers.

The denaturation of trypsin, at pH 4, by urea and guanidine.HCl is a simpler reaction than that for α-chymotrypsin. Denaturation curves for both denaturants have a smooth sigmoidal shape and do not show the presence of any intermediates in the transition region. Guanidine.HCl is more effective than urea in unfolding the enzyme, as is to be expected.

There are two methionine residues in trypsin at position 92 and 166. The residues are numbered according to the trypsinogen sequence (Dayhoff, 1969). As the two methionine resonances in the spectra of trypsin in 8 M urea and 6 M guanidine.HCl are equal in area, the simplest explaination is that one peak corresponds to methionine 92 and the other to methionine 166. The upfield chemical shift of one of the methionine resonances could possibly be due to a hydrophobic interaction between the methyl group of methionine 166 and the face of the aromatic ring of phenylalanine 167. It is unlikely that the upfield chemical shift is caused by the presence of a nearby negatively

charged group as McDonald & Phillips (1969) observed a single broad methionine peak for trypsin in 6 M guanidine.HCl at pH 7 over the temperature range 40-80°C. As the formation of a hydrophobic bond is an endothermic process the bonding would be more stable at higher temperatures. This would cause a fall in the mobility of the methionine 166 residue and a consquent broadening of its resonance to such an extent that it obscures the downfield peak. If methionine 166 is interacting with phenylalanine 167, this would have less effect on the conformation of the unfolded molecule than if residues widely spaced in the sequence of the molecule were involved.

The double methionine peak is present throughout the transition and also in the denatured protein. This can be interpreted by a denaturation mechanism involving a single step transition between the native enzyme and a denatured enzyme in which a noncovalent interaction is present. The denatured conformation cannot be regarded as a random coil, within the limits imposed by disulphide bonds, because of this residual interaction. Bradbury & King (1969) have shown that the use of stronger denaturants, such as dichloroacetic acid or trifluoroacetic acid, unfolds trypsin further than urea or guanidine.HCl as only one methionine peak was visible in spectra of trypsin in these stronger denaturants.

As Hopkins & Spikes (1968) have shown that interconversion between the native and denatured forms of trypsin is slow, F may be equated to α, the fraction of

unfolded molecules, for denaturation by both urea and guanidine.HCl.

The denaturation of trypsin is reversible at pH 4 so it appears that, unlike in α-chymotrypsin, protonation of carboxyl groups is not necessary to obtain the renatured native enzyme.

The presence of the double methionine peak, in the transition region, during the denaturation of ribonuclease and S-methyl-methionine 29-ribonuclease shows that an intermediate conformation exists as there is no methionine peak in the spectrum of the native protein and only one peak in the spectrum of the denatured protein. The chemical shift of the downfield methionine peak is the same as that of the single peak in the completely unfolded conformation. The upfield chemical shift of the other methionine peak can only be caused by the interaction of a methionine methyl group with the face of an aromatic ring as carboxylate anions would be protonated by the formic acid. The width of the downfield peak is 3 Hz and that of the upfield peak is 5 Hz (at 220 MHz). This broadening would be expected if the upfield methionine was interacting with another group. The width and chemical shift of the downfield peak is that expected for a normal methionine peak in a denatured protein (Bradbury & King, 1969).

The major step in the denaturation curve of S-methyl-methionine 29-ribonuclease occurs at 5% d_2-formic acid. This is followed by a smaller transition at about 45% d_2-formic acid, showing that denaturation by d_2-formic

acid is a multi-step process. Bradbury & King (1972) found that, in ribonuclease, the major transition occurred at 8% d_2-formic acid. This indicates that methylation of methionine 29 slightly destabilises the native enzyme conformation. In 100% d_2-formic acid the F values fall slightly as the enzyme aggregates in this solvent (Josefsson, 1958). The denaturation of S-methyl-methionine 29-ribonuclease is reversible up to an acid concentration of at least 35%. Bradbury & King (1972) have shown that denaturation of ribonuclease is reversible up to a concentration of 60% formic acid.

Figure 6.6 shows that the only differences in the spectra of native ribonuclease and S- methyl-methionine 29 ribonuclease are the presence of the S-methyl methionine peak and a sharpening of the phenylalanine peak in the spectra of the modified enzyme. This would indicate that a phenylalanine residue is forced to the surface of the enzyme when methionine 29 is methylated. This residue is probably phenylalanine 46 whose aromatic ring is 4 Å away from the methyl group of methionine 29. The distance has been calculated from the coordinates for the atoms in ribonuclease-S (Wyckoff et al, 1970), as the coordinates for ribonuclease-A have not been published.

Ribonuclease-A contains four methionine residues at position 13, 29, 30 and 79. One of these methionine residues is interacting with an aromatic ring during the denaturation of the enzyme by formic acid. The NMR spectra of the modified enzyme show that methionine 29 is not the

residue involved. Thus the upfield methionine resonance can be assigned to one of the remaining residues at position 13, 30 or 79. One can calculate the distance between the methionine methyl groups and the nearest aromatic rings from the coordinates for ribonuclease-S given by Wyckoff et al (1970). Of the three residues only methionine 13 has an aromatic group, histidine 12, adjacent to it. The distance between the imidazole ring and the methionine methyl group is 8 Å. The nearest aromatic ring to methionine 13 is that of phenylalanine 8 which is 4 Å from the methionine methyl group. The aromatic group of phenylalanine 46 is 4.6 Å from the methionine 30 methyl in the native enzyme. However, the position of this group has been altered by methylation of methionine 29 and it is impossible to determine the distance between it and the methionine 30 methyl in the modified enzyme. The nearest aromatic groups, for methionine 79, are those on tyrosine 73 and phenylalanine 120 and they are both 8 Å from the methionine 79 methyl group. All other aromatic groups are at least 10 Å from the three methionine methyl groups.

The most probable cause of the upfield methionine peak, on the basis of distance measurements, is an interaction between the methyl group of methionine 13 and the aromatic ring of phenylalanine 8. Implicit in this assignment is the assumption that the methionine residue responsible for the upfield peak will interact with the nearest aromatic residue. If this assumption is incorrect then any one of the three methionine residues, 13, 30 or 79,

could be the cause of the upfield methionine peak by being forced next to a distant aromatic group by noncovalent bonding in some other part of the molecule. The only thing known with any certainty is that the upfield peak is not caused by a hydrophobic interaction between the methyl group of methionine 29 and the face of an aromatic ring.

CHAPTER 7

THE STRUCTURE OF 51A PROTEIN

This is a joint project with Dr. A. Reisner, Division of Animal Genetics, C.S.I.R.O., Epping.

7 A INTRODUCTION

Coating the surface of the ciliated protozoan, Paramecium aurelia, are a closely related family of proteins called immobilisation antigens.* Protein 51A is a member of this group of proteins from Paramecium stock 51. An individual Paramecium is capable of synthesising over a dozen of these proteins, but normally only one can be detected at a given time. It is known that only one structural gene is involved in the synthesis of an immobilisation antigen, thus the system involves multi-cistronic** regulation and an understanding of this system may prove valuable in studies of the regulation of protein synthesis.

Reisner et al (1969 a & b) has studied the structure of several of these proteins to obtain information

---

*These proteins are called immobilisation antigens because the addition of antibody to a suspension of Paramecium aurelia causes them to loose their mobility due to the formation of the antibody-antigen complex.

** A cistron is a unit of DNA codeing the amino acid structure of one of the surface proteins.

that may be of value in the analysis of the control system. The molecular weight of 51A protein, determined by equilibrium centrifugation studies, is 301,500$\pm$4,500 g/mole. This value, combined with amino acid analyses, indicates that the protein consists of 2,930 amino acid residues. Ultracentrifugation, starch gel electrophoresis and viscosity studies indicate that the protein consists of a single polypeptide chain. Protein 51A is the largest known monomeric globular protein and for this reason its structure in solution is worth studying.

In this chapter, an NMR study on the native protein and its denaturation by urea was undertaken to obtain information about the structure of the protein.

7 B EXPERIMENTAL

Samples of 51A protein were supplied by Dr. A. Reisner. The preparation and purification of the protein is described by Macindo & Reisner (1967) and Reisner et al (1969 b). Most of the spectra were obtained at 60 MHz on 10% (w/v) solutions in D_2O at pH meter reading 4. For the progressive urea denaturation study, weighed amounts of urea were added to a solution initially at 10% protein concentration. The pH was readjusted to 4 after each addition and the spectrum was obtained. Measurements of peak heights were adjusted to compensate for dilution upon adding urea

7 C RESULTS

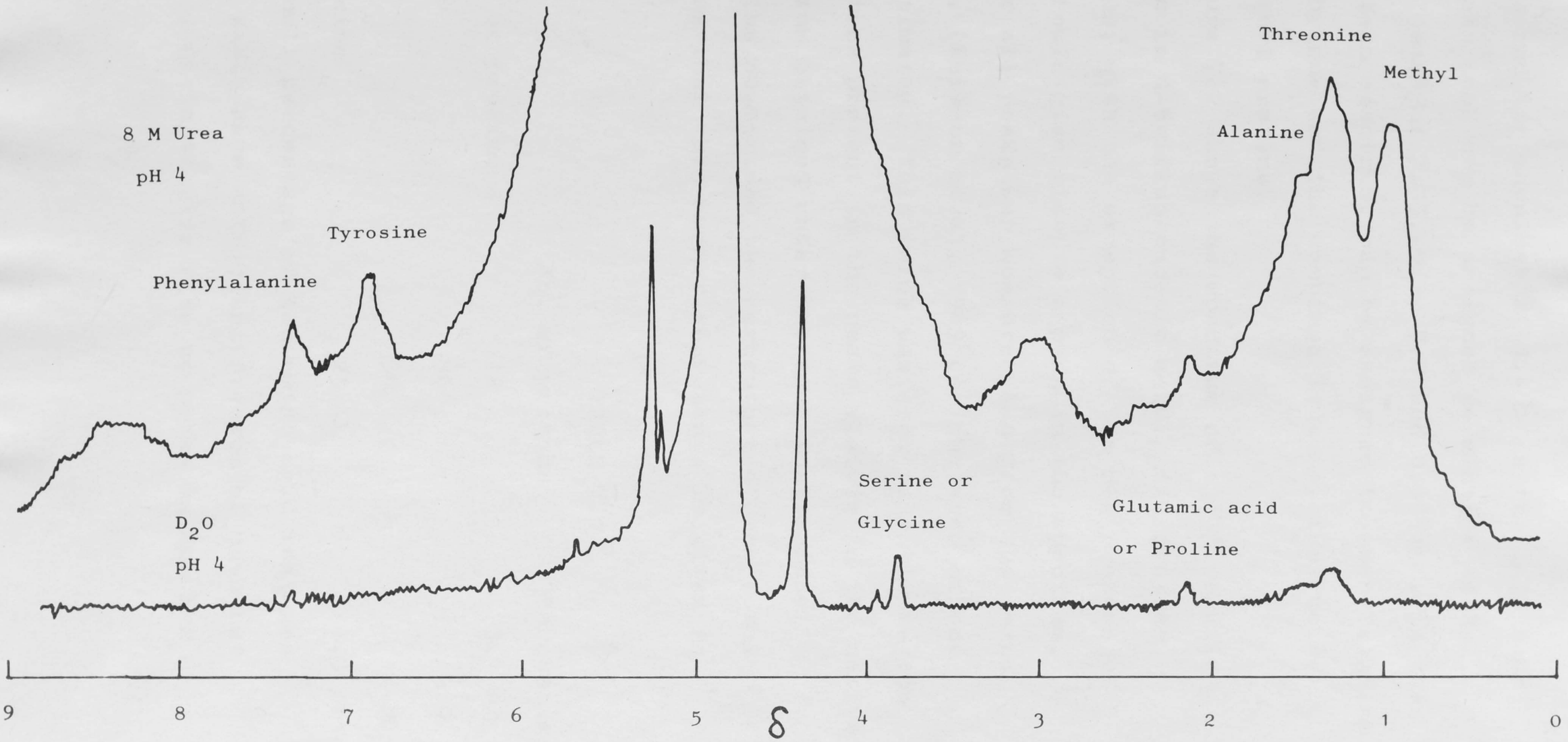
The 60 MHz spectra of 51A protein in D_2O and 8 M

urea, at pH 4, are shown in Figure 7.1. The spectrum of the native protein is extremely broad with only three peaks visible above the baseline, peak one at 3.8 δ, peak two at 2.1δ and peak three at 1.25 δ. Reisner et al (1969 a) have shown that the protein does not aggregate at 2.5% concentration and the 10% solutions used in the NMR work were not very viscous, so it is reasonable to assume that the protein does not aggregate at 10%. The broadening must be due to the decreased mobility of the protons in the protein due to the large molecular weight. Protons giving rise to peaks in the native protein spectrum must have local motional freedom independent of the motion of the molecule as a whole.

The peak at 3.8 δ can be assigned to serine or glycine $-CH_2-$ protons as this is the resonance position for these protons. (McDonald & Phillips, 1969). The peak at 2.1δ is at the correct resonance position for methionine $-CH_3$ protons. However, the position of this peak could not be shifted by methylating the native protein with methyl iodide or by oxidising it with H_2O_2. Treatment of the protein, in an 8 M urea solution, with H_2O_2 resulted in the removal of the peak in one experiment, but the experiment could not be repeated. It seems unlikely that this peak is due to methionine methyl protons. McDonald and Phillips (1969) state that the β-CH_2 protons of proline and glutamic acid also give rise to resonances at this position. The peak at 2.1 δ could be due to one or both of these amino acid residues. The peak at 1.25δ can be assigned to threonine

Figure 7.1

51A Protein



methyl protons and the shoulder at 1.4 δ on the left hand side of the main peak can be assigned to alanine methyl protons. The peaks at 7.35, 6.9, 3.0 and 0.95 δ , in the denatured protein spectrum, can be assigned to phenylalanine, tyrosine, methylene and the combined leucine, isoleucine and valine methyl protons.

Figure 7.2 shows the spectrum of a 10% solution of 51A protein in d-trifluoroacetic acid. All protons on carbon atoms, with the exception of the C-2 protons of tryptophan, should give rise to a peak in the spectrum. The area under all peaks was measured and from the amino acid analysis, (Reisner et al, 1969 c), the area of one proton was estimated. This value was used to estimate the number of protons present in the peaks visible in the native protein spectrum obtained under the same instrumental conditions. The number and the percentage of the total of such protons that this number represents is given in Table 7.1.

TABLE 7.1

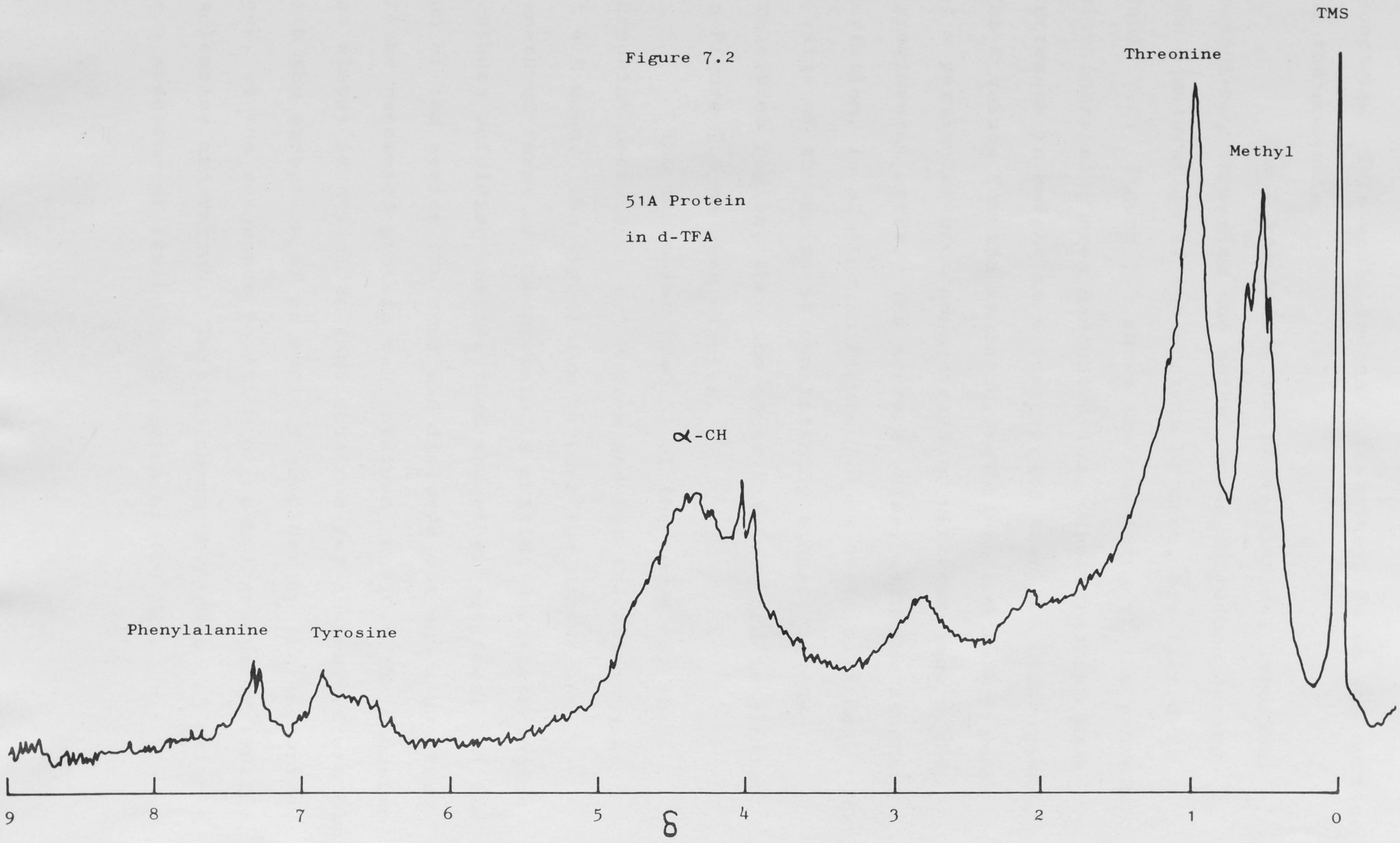
	No. of protons	Percentage
Glutamic acid or proline	12	3.4 or 11
Threonine	56	3.9
Alanine	30	2.9
Serine or glycine	25	4.3 or 6.2

A small percentage of the amino acid residues listed in the table have sufficient freedom of movement in the native protein to give rise to peaks in the NMR

Figure 7.2

51A Protein

in d-TFA



spectrum. This would indicate that they are on the surface of the molecule.

The F values for the phenylalanine, tyrosine, methylene, threonine and methyl peaks, obtained during the denaturation of the protein by urea, are listed in Table 7.2. Figure 7.3 shows the unfolding of the protein with increasing urea concentration. The threonine peak increases in height at a faster rate than the other peaks. The F values for the aromatic, methylene and methyl peaks, at a particular urea concentration, were the same within experimental error. The average value, with the standard deviation, is plotted in Figure 7.4 as vertical lines. The F value of threonine is consistently higher, in the transition region, than the other F values and is plotted in Figure 7.4 as open circles.

The mid-point (F=0.5) of the curve for the threonine peak is at 2.5 M urea and for the other peaks at 4 M urea. The transition between the native and denatured forms of the protein is multistep with threonine residues unfolding earlier than the other residues. At the end of the series the urea was dialysed out and a spectrum of the recovered protein was obtained in D_2O. The spectrum was almost identical to that obtained for the native protein with the exception of an upfield shoulder on the threonine peak, at the resonance position of methyl groups of leucine, isoleucine and valine. This is shown in Figure 7.5 which is a spectrum of the upfield region at 100 MHz.

Figure 7.3

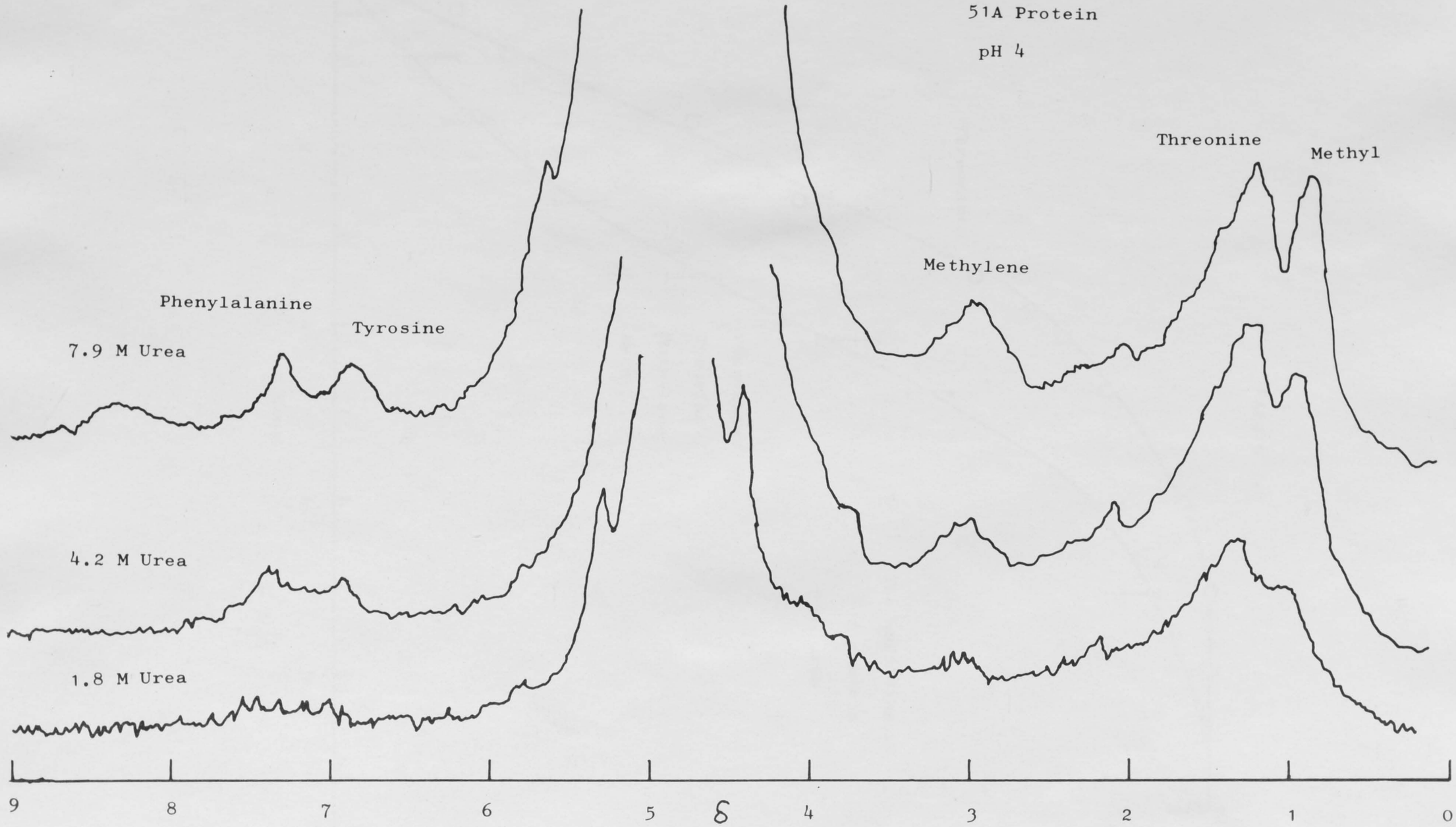

51A Protein
pH 4
Phenylalanine
Tyrosine
7.9 M Urea
4.2 M Urea
1.8 M Urea
Methylene
Threonine
Methyl
9
8
7
6
5
δ
4
3
2
1
0

Figure 7.4

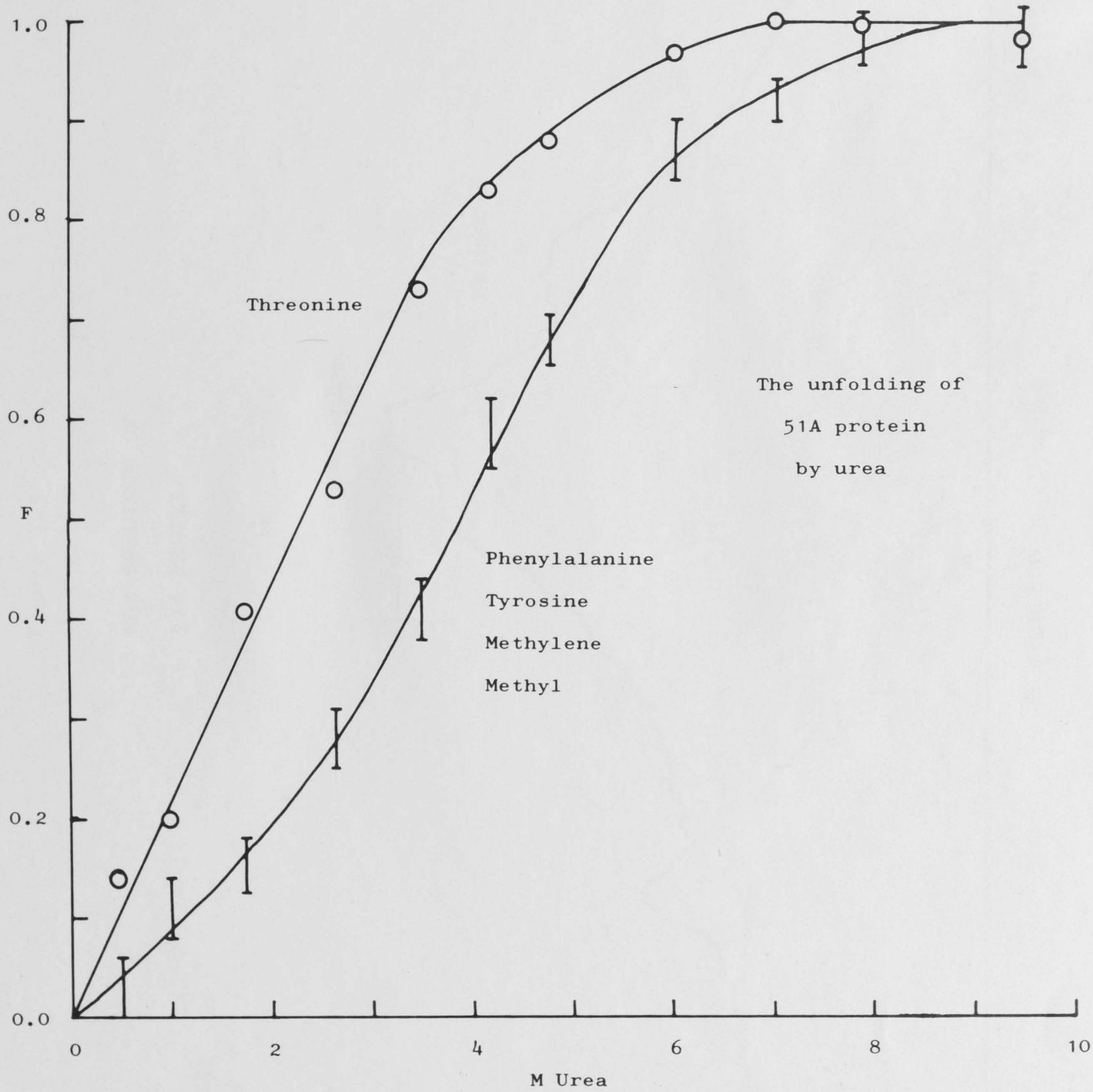

1.0
0.8
0.6
F
0.4
0.2
0.0
0
2
4
6
8
10
M Urea
Threonine
Phenylalanine
Tyrosine
Methylene
Methyl
The unfolding of
51A protein
by urea

Figure 7.5

100 MHz SPECTRUM OF

51A PROTEIN

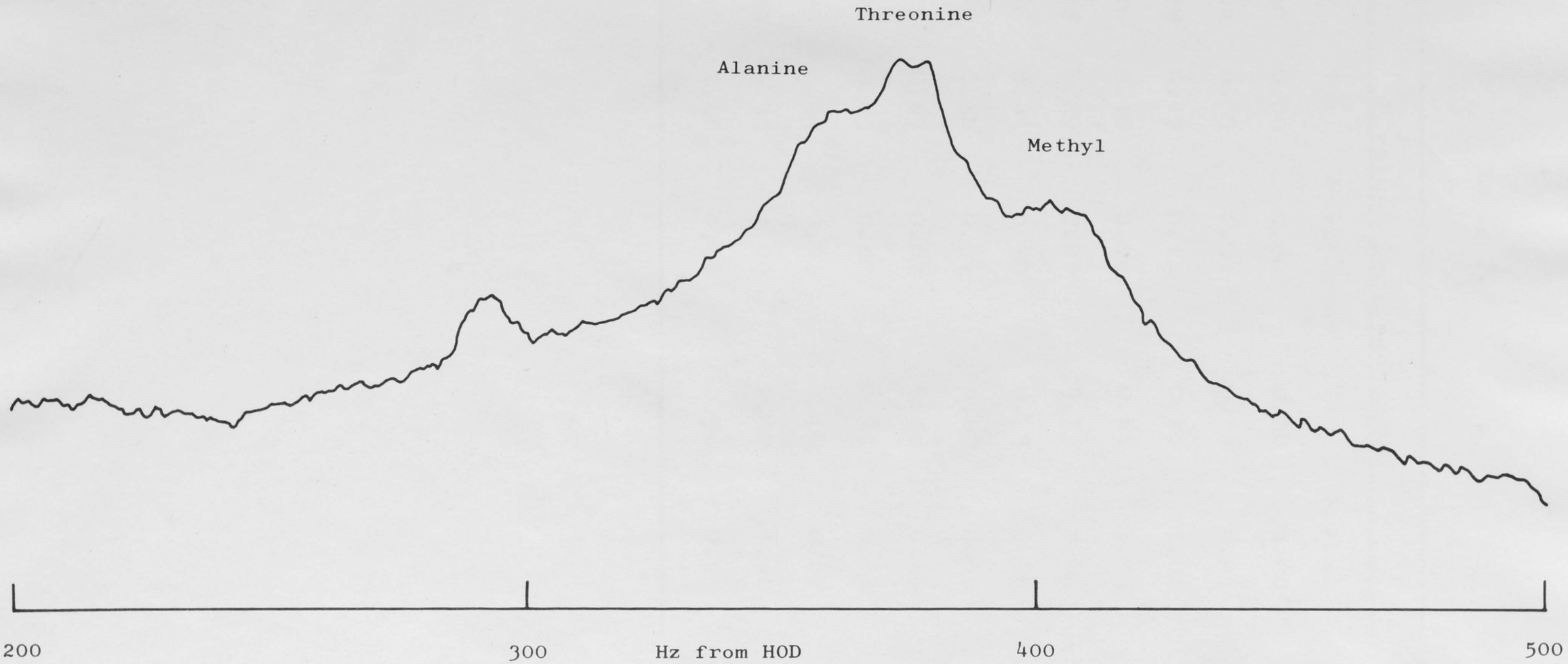


Table 7.2

Urea conc.	F values for resonances				
(M)	Phe	Tyr	Methylene	Thr	Methyl
0.0	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.09	0.14	0.05
1.0	0.09	0.17	0.11	0.20	0.06
1.8	0.16	0.18	0.14	0.41	0.13
2.7	0.35	0.29	0.25	0.53	0.22
3.5	0.46	0.41	0.40	0.73	0.36
4.2	0.62	0.58	0.51	0.83	0.62
4.8	0.75	0.67	0.63	0.88	0.65
6.1	0.92	0.85	0.87	0.97	0.84
7.1	0.95	0.92	0.92	1.00	0.87
7.9	0.99	0.93	1.00	0.99	1.00
9.5	1.00	1.00	0.98	0.98	0.95

7 D DISCUSSION

Protein 51A has a low ratio of nonpolar to polar amino acids, a high content of cysteine (11 moles %), alanine (12 moles %) and hydroxylamino acids (26 moles %) particularly threonine (16 moles %).

Reisner et al (1969 c) examined negatively stained preparations of the protein in an electron microscope. The only structural material observed in these preparations were discs of about 175 Å diameter. Reisner et al have proposed a model of the protein on the basis of this observation and the amino acid composition. The assumption is made that all hydrophobic residues are in the interior of the molecule and the hydrophilic residues are on the exterior forming a layer 4 Å thick. The dimensions of oblate and prolate ellipsoids that would satisfy the amino acid composition can then be determined by using an equation developed by Jennings (1968). Solving the Jennings equation yields dimensions for an oblate ellipsoid of 210 x 15 Å in fairly good agreement with the result from electronmicroscopy. Further calculation has shown that it is possible for the polar amino acids to form a monolayer 170 Å in diameter by 25 Å thick that would just surround a circular disc of nonpolar amino acids two residues thick.

The NMR data would be consistent with a model that assumed the protein was made up of a hydrophobic core containing phenylalanine, tyrosine, leucine, isoleucine and valine residues surrounded by a hydrophilic sheet containing serine, threonine, glutamic acid or proline

and some alanine residues. The residues in the interior of the molecule could only move as the molecule rotates as a whole and would give rise to very broad peaks due to dipole-dipole interactions. This would explain the non-appearence of peaks from these residues in the NMR spectrum of the native protein. Residues on the surface of the molecule would have some local motional freedom, independent of the molecule as a whole, provided they were not involved in noncovalent interactions. These residues would give rise to peaks in the native protein spectrum.

The rapid increase in F values for threonine with added urea suggests that threonine residues predominate in a part of the molecule that unfolds ahead of the rest of the molecule. This segment can only be at the surface of the molecule as residues in the interior cannot unfold at a faster rate than that of the surrounding residues. The 3.8 δ peak is obscured by the urea-HOD peak and the alanine residues do not give rise to a distinct peak in the NMR spectrum. Thus the F values of these peaks could not be measured. The slower increase in F values, with added urea, for the aromatic, leucine, isoleucine and valine peaks suggests that residues giving rise to these peaks are in a stable part of the molecule,probably in the interior.

One can propose the following structure for 51A protein from the NMR results and the results of Reisner et al (1969 c). The overall structure of the protein consists of flat discs with a diameter of about 175 Å. The interior

of the molecule is made up of a hydrophobic core stabilised by the thiol bridges of cystine. This core is surrounded by a hydrophilic coat containing most of the threonine and serine residues in the form of β-pleated sheets, a structure favoured by these amino acids. Reisner et al (1969 c) found that there was little if any α-helix present in the protein. The methyl group of threonine could serve to bond the coat to the hydrophobic core while the hydroxyl groups of the two amino acids could form hydrogen bonds to the surface of the Paramecium. As Klotz (1970) has shown that hydrophobic residues occur at the surface of all globular proteins whose three dimensional structure is known, one would expect some hydrophobic residues at the surface of 51A protein. The NMR results on the native protein indicate that these would mainly consist of alanine residues.

CHAPTER 8

THE DEUTERATION OF RIBONUCLEASE-A

8 A INTRODUCTION

The assignment of the four C-2 histidine resonances observed in the NMR spectrum of ribonuclease-A in 0.1 M sodium acetate solutions, (Figure 8.1), has been the topic of a number of investigations. Meadows et al (1967) published a partial assignment for the resonances. The proton of peak 4 appears to be in a different environment from those of the other histidine residues as King & Bradbury (1971) have shown that this resonance can not be seen above pH 5.5 in the absence of acetate, and at pH 4, in the presence of acetate, the peak is 0.5 ppm upfield from the other resonances. Meadows et al assigned peak 4 to histidine 48 as Wyckoff et al (1970) have shown that this residue is partially buried in the X-ray crystallographic model.

Meadows et al (1967) also found that when the competitive inhibitor cytidine-5'-phosphate was added to a solution of ribonuclease-A, peaks 2 and 3 had their chemical shifts and widths altered. Since Crestfield et al (1963) have shown that histidine 12 and 119 are involved in the enzymatic activity, Meadows et al (1967) assigned peaks 2 and 3, which are most affected by the binding of the inhibitor, to histidine 12 and 119. However they were not able to differentiate between the two different assignments possible. The remaining peak, peak 1, was assigned to histidine 105.

The two histidine residues in the active site could be assigned,in principle, by modifying one of them

and obtaining the NMR spectrum of the modified enzyme. The residue that has been modified should have a C-2 peak with a different chemical shift or width or both shift and width from that in the unmodified enzyme. Crestfield et al (1963a) have shown that iodoacetic acid can be used to modify histidine 12 or 119 exclusively and that the two enzymes can be separated by ion-exchange chromatography. Meadows et al (1968) obtained the spectrum of these two modified enzymes but found that the presence of one modified histidine in the active site affected both peaks 2 and 3. Thus this method is of no use in the identification of the two active site histidine C-2 resonances.

Meadows et al (1968) were able to assign the histidine C-2 resonances in ribonuclease-S by exchanging the C-2 proton of histidine 12, in the S-peptide, with a deuteron. The enzyme was then reconstituted and its NMR spectrum obtained. The missing peak was assigned to histidine 12. The missing peak occurred at the position of peak 2 in the protonated enzyme. Thus Meadows et al (1968) assigned peak 2 to histidine 12 and peak 3 to histidine 119. The authors then used the same assignment in ribonuclease-A. Implicit in this assignment is the assumption that the two active site histidine resonances maintain the same relative position in both ribonuclease-A and S. As the chemical shifts of the resonances in the two enzymes are different, this assumption might not be justified.

King & Bradbury (1971) have shown that the apparent pK's, in D_2O, of histidine 105, 12 and 119 are 6.4, 5.8 and

5.2 respectively. Richards & Wyckoff (1971) have said that it is difficult to understand why histidine 12 has a pK so near to normal and histidine 119 a low pK as, in the crystal structure, histidine 12 is in the active site cleft of the enzyme while histidine 119 is pointing out into the solvent. One might expect the pK's to be the other way around.

In this chapter the possibility of differentially exchanging the C-2 protons of histidine 12 and 119 will be investigated. If this can be accomplished, an unambiguous assignment of the two resonances can be made by splitting the deuterated ribonuclease-A with subtilopeptidase-A, to form ribonuclease-S, and then obtaining the NMR spectrum of the S-protein, containing residue 119, and the S-peptide, containing residue 12.

8 B EXPERIMENTAL

A 1% solution of ribonuclease-A, in D_2O, was prepared by dissolving 500 mg. of the Worthington enzyme in an appropriate volume of D_2O. The pH of the solution was adjusted to pH(meter reading) 8.5 and the sample was placed in a waterbath set at 37°C. At appropriate intervals some of the sample was removed and the solvent lyophilised off. A 10% solution of the deuterated enzyme was prepared in either D_2O or 0.10 M acetate/D_2O at about pH(meter reading) 5.7.

Most of the NMR spectra were obtained on D_2O solutions as, although the peak due to histidine 48 can not be seen at pH 5.7, there is a larger difference in chemical

shift between the three visible peaks than there is in acetate solutions. This makes it easier to measure the area of the peaks. At the end of spectrum accumulation, for D_2O solutions, the pH was lowered to 4.0 and another spectrum was obtained at this pH so that the area of the peak due to the C-2 proton of histidine 48 could be measured.

The relative number of protons contributing to each C-2 histidine resonance was determined by comparing the weight of the tracing of the histidine peak to the weight of the tracing of the aromatic peak.

Ribonuclease-S was prepared according to the method of Richards & Vithayathil (1959). 400 mg. of ribonuclease-A was dissolved in 3 ml. of 0.10 M KCl solution, the pH was adjusted to 8.0 and the temperature was lowered to 5°C. 1.6 mg. of subtilopeptidase-A (in 1.6 ml. of H_2O) was added to the ribonuclease-A solution. The pH was kept at 8.0 by the addition of 0.10 M NaOH. After 3 hours the reaction was stopped by lowering the pH to 3.0. The digest was placed on a 4 x 75 cm. column of IRC-50 ion-exchange resin equilibrated with 0.2 M phosphate buffer pH 6.35. The sample was eluted with the phosphate buffer at a flow rate of 60 mls/hr. and collected in 20 ml. fractions. A LKM Uvicord II Ultraviolet Absorptiometer was used to continuously measure the optical density of the effluent at 280 mμ. Fractions containing ribonuclease-S were pooled, the phosphate removed by dialysis and the protein isolated by lyophilisation.

A 1% solution of ribonuclease-S was prepared in a

centrifuge tube and the temperature of the solution was lowered to 0°C. An amount of 20% trichloroacetic acid solution equal to 20% of the volume of the ribonuclease-S solution was slowly added, with stirring, at 0°C. The ribonuclease-S protein precipitated out of the solution when it was slowly warmed up to room temperature. The S-protein was packed down by centrifugation and the supernatant, containing the S-peptide, was removed. The S-protein was dissolved in the same volume of water as the original ribonuclease-S sample and the above treatment with trichloroacetic acid was repeated. The two supernatant fractions were combined and the trichloroacetic acid was removed by extensive extraction with ether. Towards the end of the extraction procedure, small amounts of 0.1 M HCl were added to remove the last traces of the trichloroacetic acid. The S-peptide was isolated from the aqueous solution by lyophilisation. The S-protein was dissolved in water and the trichloroacetic acid was removed by dialysis against 10^{-3} M HCl at 4°C for two days. At the end of this time the solvent was lyophilised off and the S-protein isolated.

NMR spectra of the S-protein were obtained on 10% solutions in 0.1 M sodium acetate/D_2O at pH 5.9. The spectra of the S-peptide were obtained on 2% solutions in 0.1 M sodium acetate/D_2O pH 7.0 at 10°C.

8 C RESULTS

The downfield region of the 100 MHz spectra of ribonuclease-A and 6 days deuterated, at pH 8.5, ribonuclease-A

in 0.1 M sodium acetate/D_2O is shown in Figure 8.1. The bottom spectrum is of the aromatic region of 6 days deuterated ribonuclease-A at pH 5.9. It can be seen that the only C-2 histidine peaks visible in the spectrum are peaks 3 and 4. The C-2 protons of the histidine residues giving rise to peaks 1 and 2 have exchanged with deuterons to such an extent that the peaks are not visible above the baseline. The upper spectrum shows the four histidine C-2 resonances of protonated ribonuclease-A at pH 5.7. The two spectra show that the chemical shift of the C-2 histidine peaks is very sensitive to the pH and that it is difficult to find a pH where all four peaks are well separated. For this reason most of the spectra were obtained in D_2O solutions where the three histidine C-2 peaks, peaks 1, 2 and 3, that are visible in this solvent at pH 5.6 are well separated. This makes it easier to measure the area of each histidine C-2 peak.

Figure 8.2 shows the histidine C-2 region of the 100 MHz spectra of ribonuclease-A in D_2O at pH 5.6 for a number of samples deuterated at pH 8.5 for various periods of time. Peak 4 is not visible at this pH. It can be seen that the area of peaks 1 and 2 falls at a faster rate than does the area of peak 3.

At the end of spectrum accumulation at pH 5.6, for D_2O solutions, the pH was adjusted to 4.0 and the spectrum was obtained at this pH to enable the area of the remaining histidine C-2 peak, peak 4, to be measured. Figure 8.3 shows the downfield region of the 100 MHz spectra of 4 days

Figure 8.1

Ribonuclease-A

in 0.1 M acetate

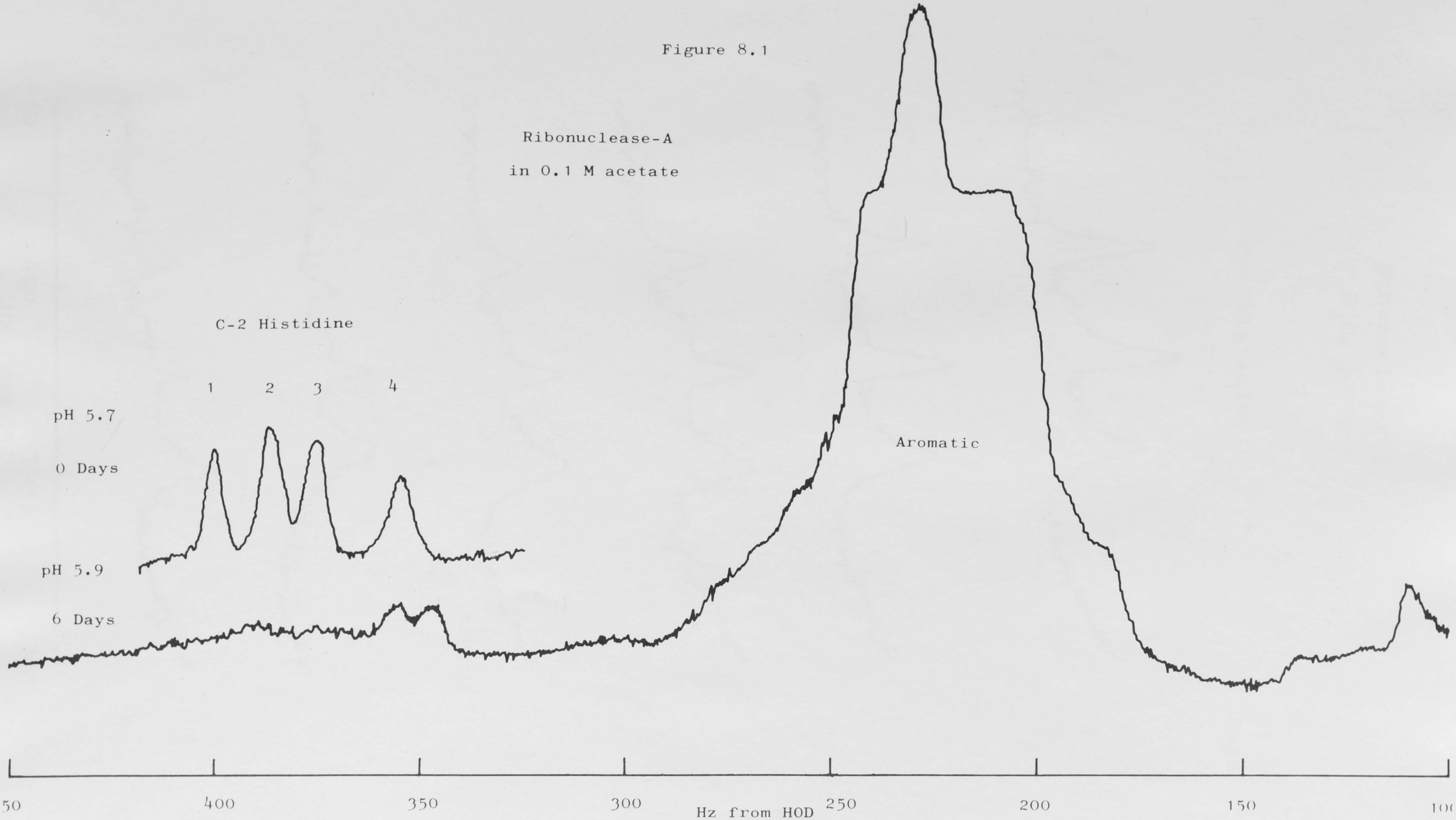


Figure 8.2

Ribonuclease-A

D_2O, pH 5.6

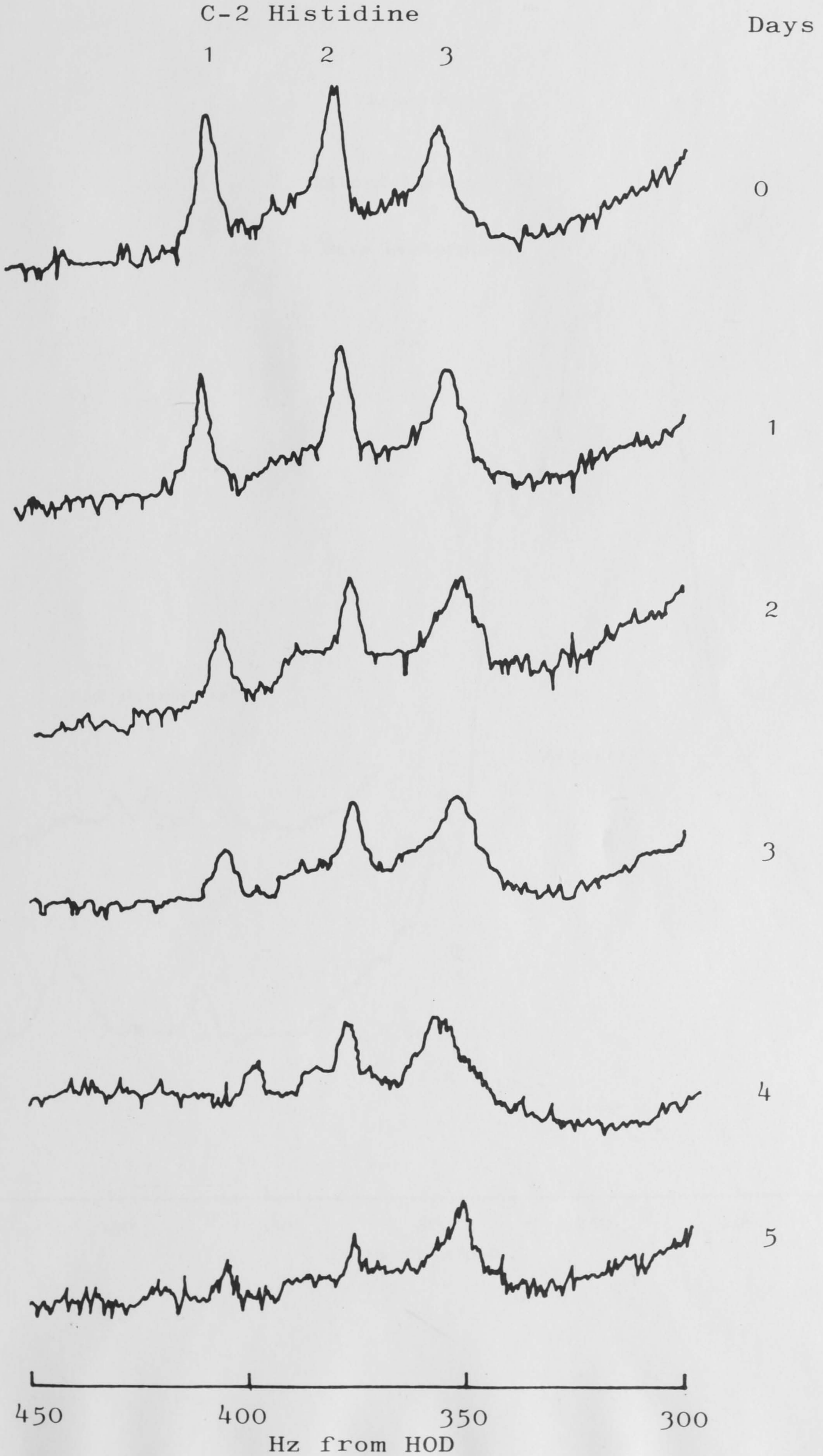
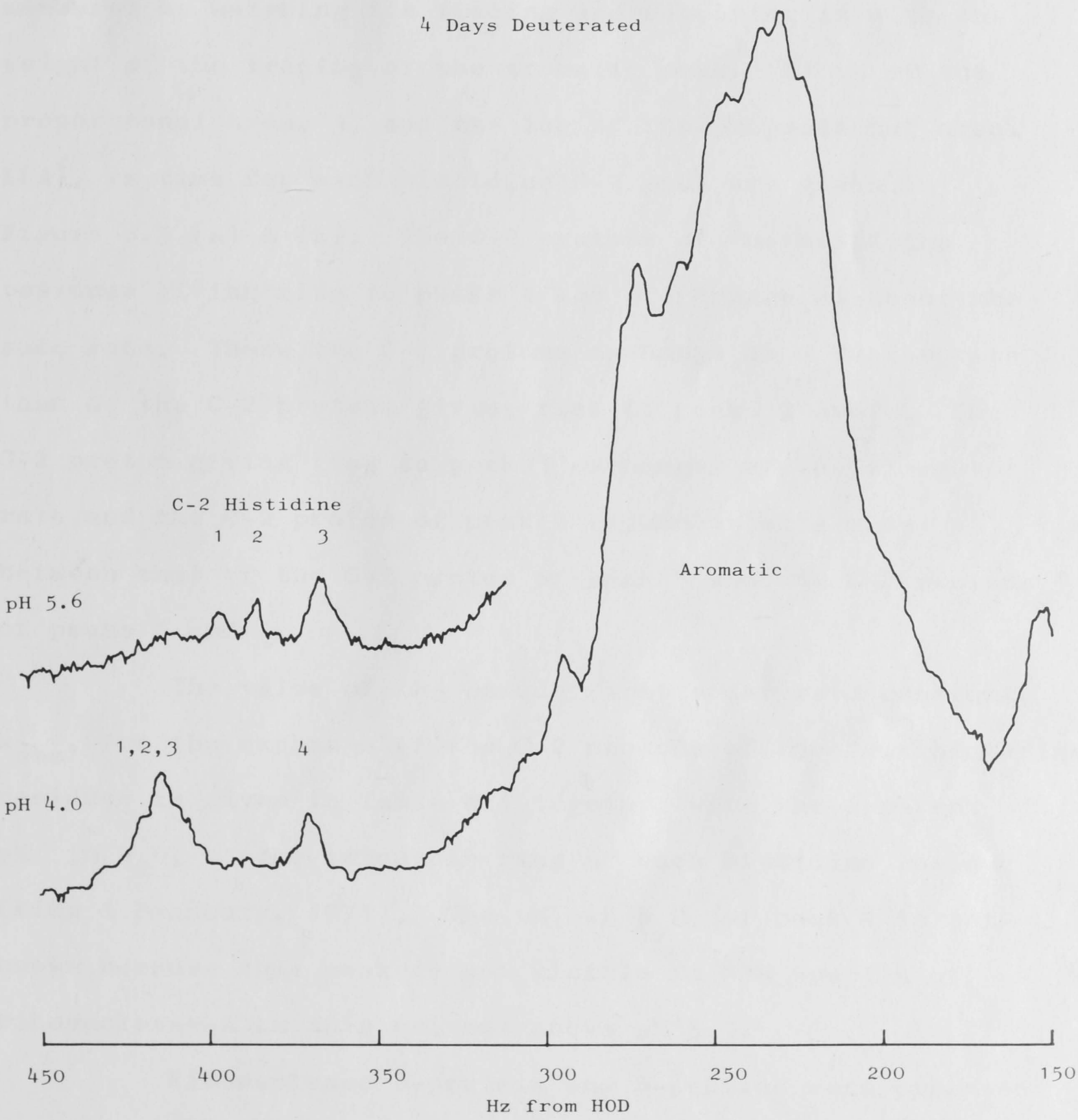


Figure 8.3


Ribonuclease-A
4 Days Deuterated
C-2 Histidine
1 2 3
pH 5.6
1,2,3
4
pH 4.0
Aromatic
450
400
350
300
250
200
150
Hz from HOD

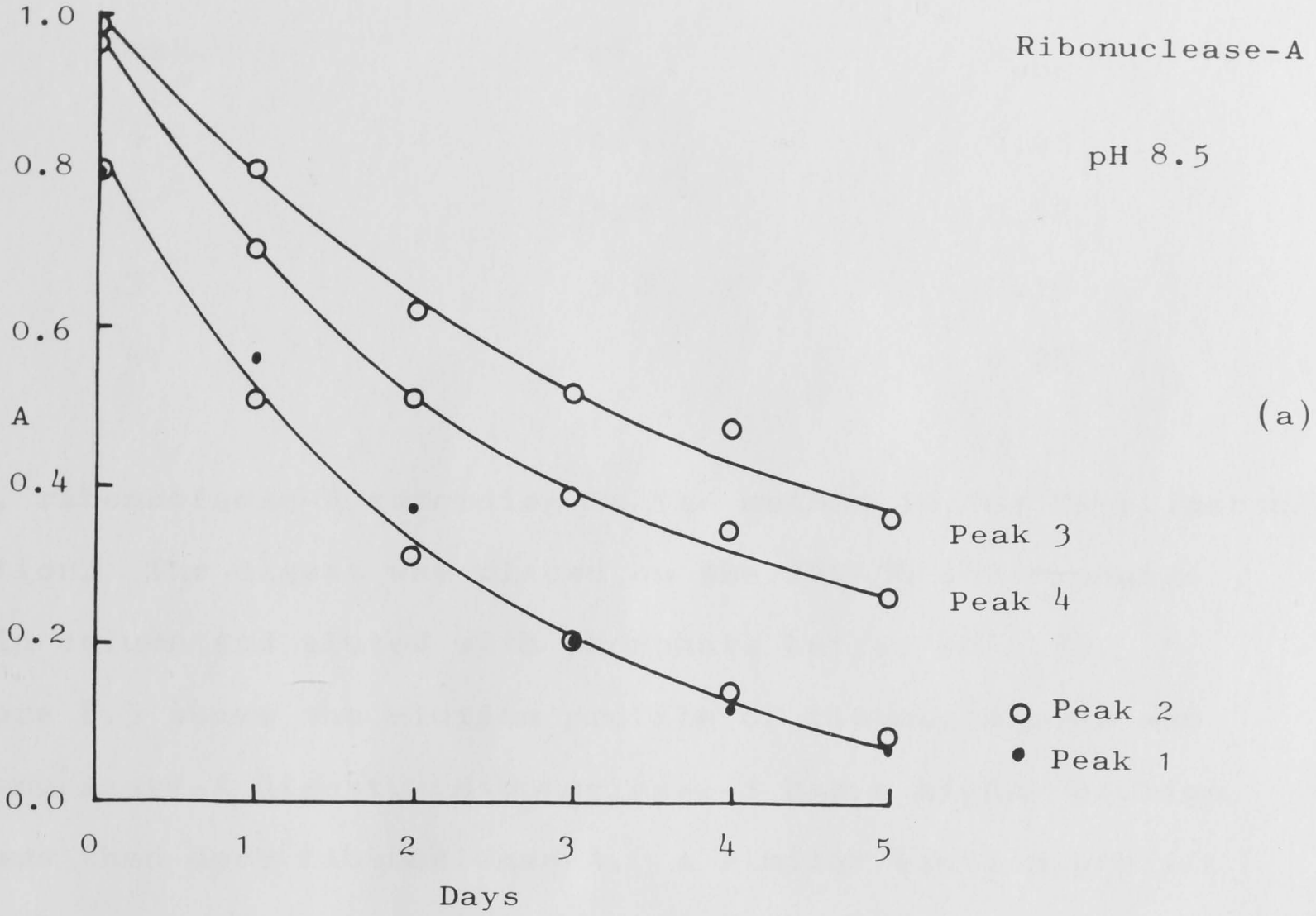
deuterated, at pH 8.5, ribonuclease-A in D_2O at pH 4.0 and 5.9. The bottom spectrum shows the aromatic region at pH 4.0. Histidine C-2 peaks 1,2 and 3 have merged into one peak and peak 4 is present upfield from the combined peak. The upper spectrum shows histidine C-2 peaks 1, 2 and 3 at pH 5.9. Peak 4 is not visible at this pH.

The area of each of the histidine C-2 peaks was measured by weighing its tracing and comparing it with the weight of the tracing of the aromatic peak. Plots of the proportional area, A, and the log of the proportional area, L(A), vs time for each histidine C-2 peak are given in Figure 8.5 (a) & (b). The C-2 protons of the histidine residues giving rise to peaks 1 and 2 exchange at about the same rate. These two C-2 protons exchange at a faster rate than do the C-2 protons giving rise to peaks 3 and 4. The C-2 proton giving rise to peak 3 exchanges at the slowest rate and the C-2 proton of peak 4 exchanges at a rate between that of the C-2 proton of peak 3 and the C-2 protons of peaks 1 and 2.

The value of the pseudo first order rate constant, k_{obs}, for the exchange of the C-2 protons of the four histidine residues is given in Table 8.1 together with the apparent pK, in D_2O, of the imidazole ring of each histidine residue (King & Bradbury, 1971). The pK, in D_2O, of peak 4 is not known because this peak is not visible in NMR spectra of ribonuclease-A in this solvent above pH 5.5.

Ribonuclease S-protein and S-peptide were prepared from protonated ribonuclease-A and 5 days deuterated, at pH

Figure 8.4


Ribonuclease-A
pH 8.5
1.0
0.8
0.6
A
0.4
0.2
0.0
0
1
2
3
4
5
Days
Peak 3
Peak 4
Peak 2
Peak 1
(a)

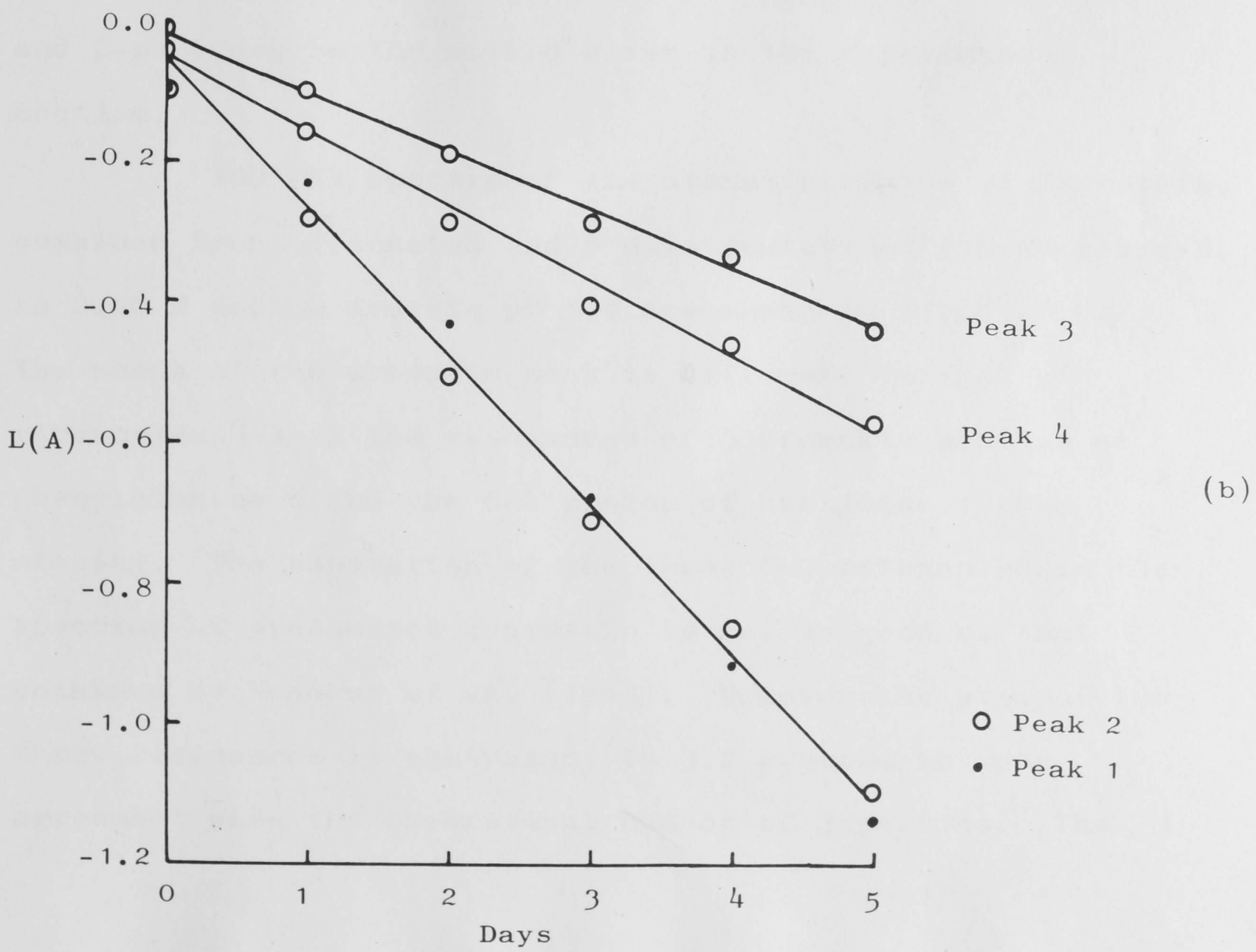

0.0
-0.2
-0.4
L(A)-0.6
-0.8
-1.0
-1.2
0
1
2
3
4
5
Days
Peak 3
Peak 4
Peak 2
Peak 1
(b)

TABLE 8.1

Peak	pK	k_{obs}
1	6.4	0.48
2	5.8	0.48
3	5.2	0.19
4		0.25

8.5, ribonuclease-A according to the method in the Experimental Section. The digest was placed on the IRC-50 ion-exchange resin column and eluted with phosphate buffer pH 6.35. Figure 8.5 shows the elution profile of ribonuclease-A and ribonuclease-A digest. Ribonuclease-S has a higher elution volume than does ribonuclease-A. A similar elution profile was observed by Richards & Vithayathil (1959). Ribonuclease-S was isolated and separated into its components, S-protein and S-peptide, by the method given in the Experimental Section.

100 MHz spectra of the aromatic region of S-protein, obtained from protonated and 5 days deuterated ribonuclease-A, in 0.10 M sodium acetate pH 5.9 are shown in Figure 8.6. The shape of the aromatic peak is different to that of ribonuclease-A as the resonances of 5 aromatic protons of phenylalanine 8 and the C-4 proton of histidine 12 are missing. The separation of the three C-2 resonances in the spectrum of protonated S-protein is not as good as that obtained by Meadows et al, (1968). However the area of the three resonances is equivalent to 3.2 protons in good agreement with the theoretical number of 3 protons. The

Figure 8.5

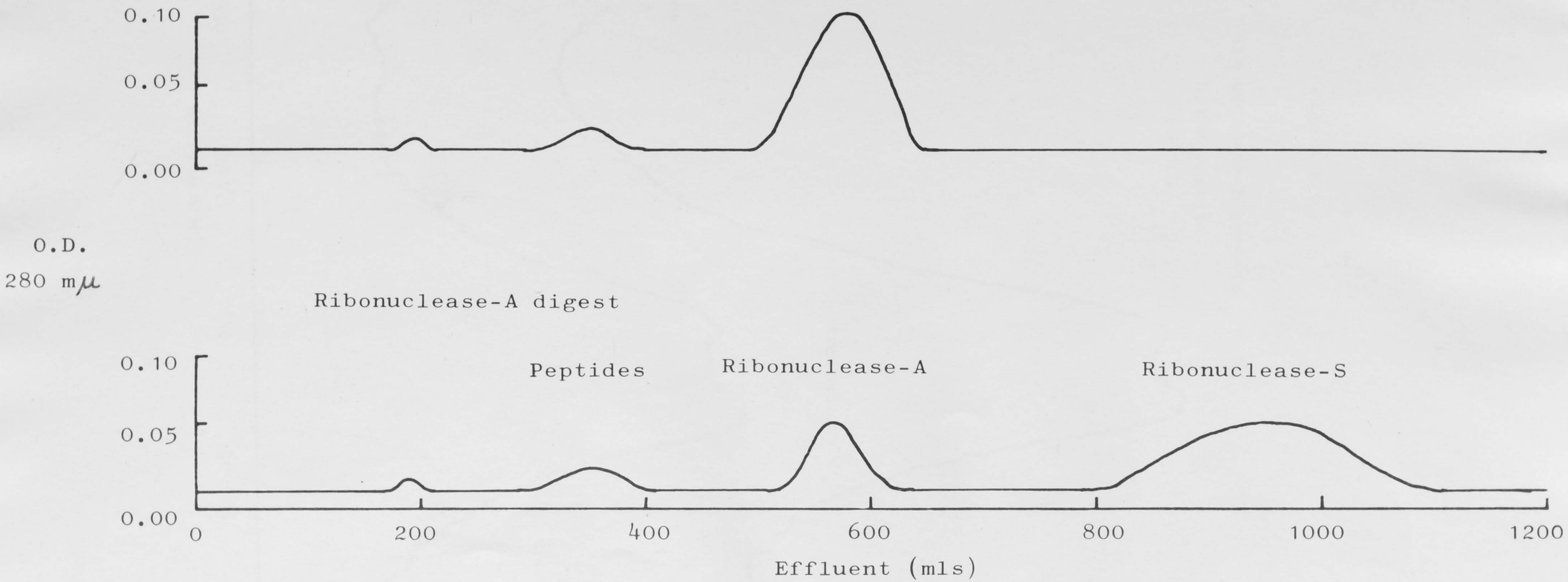
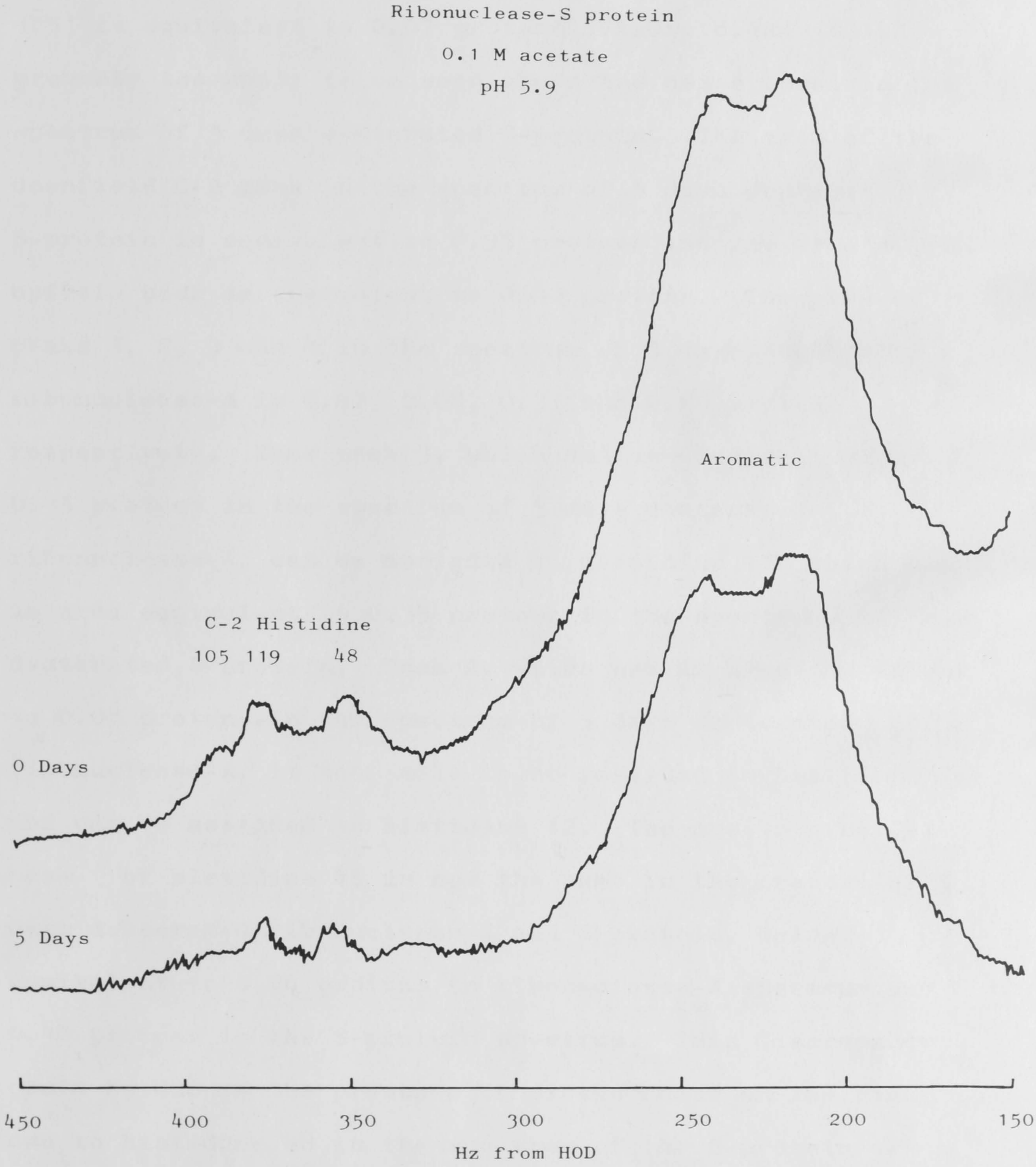

4 x 75 IRC-50 column
0.2 M phosphate buffer, pH 6.35
Ribonuclease-A
0.10
0.05
0.00
O.D.
280 mμ
Ribonuclease-A digest
Peptides
Ribonuclease-A
Ribonuclease-S
0.10
0.05
0.00
0
200
400
600
800
1000
1200
Effluent (mls)

Figure 8.6


Ribonuclease-S protein
0.1 M acetate
pH 5.9
Aromatic
C-2 Histidine
105 119 48
0 Days
5 Days
450
400
350
300
250
200
150
Hz from HOD

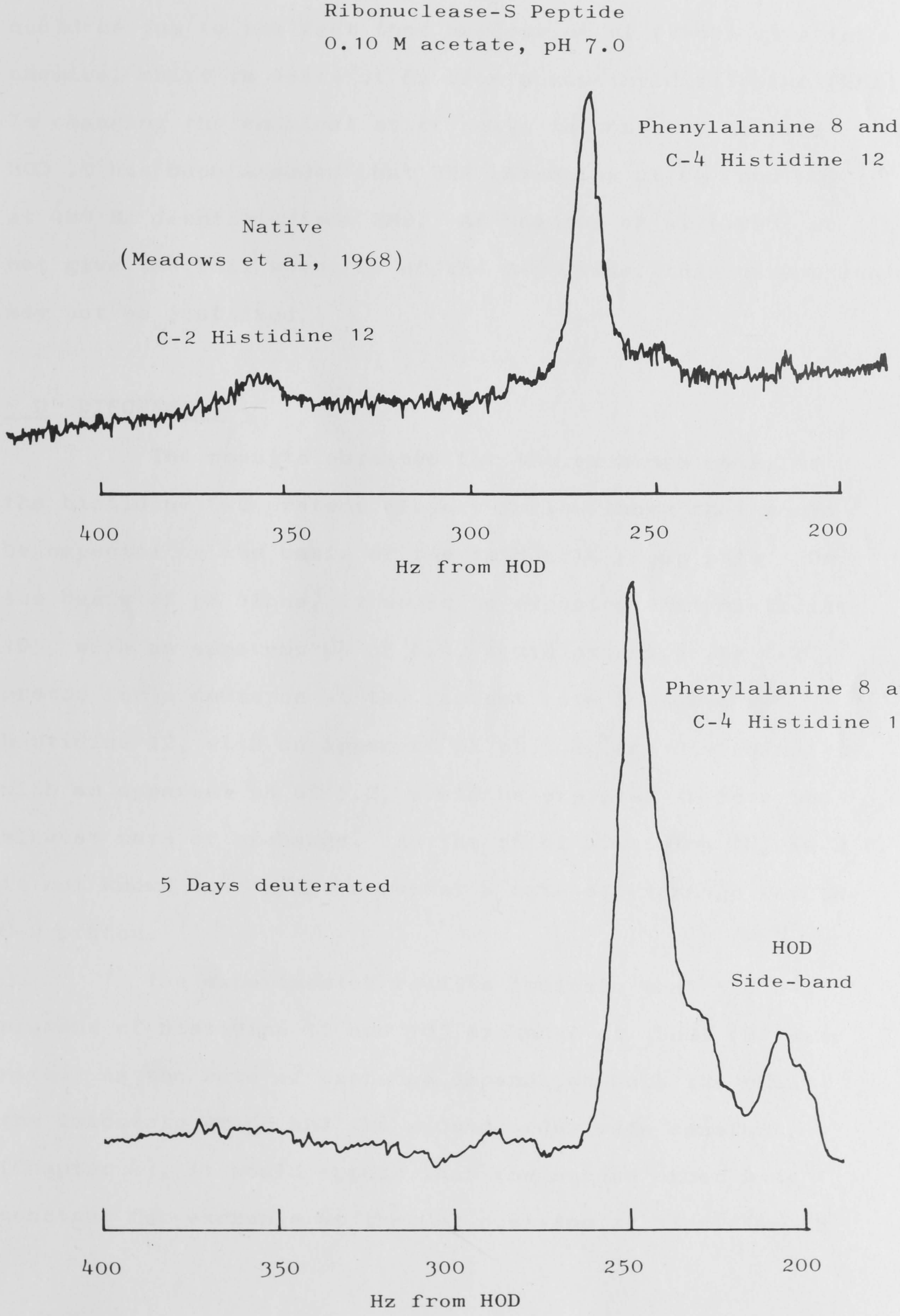
assignment of the three C-2 resonances is from Meadows et al (1968). The spectrum of S-protein obtained from 5 days deuterated ribonuclease-A shows two histidine C-2 resonances, one at the position for histidine 119 and the other at the position for histidine 48. As the area of peak 1 (histidine 105) is equivalent to 0.07 protons (Figure 8.4a) it is probably too small to be seen above the noise level in the spectrum of 5 days deuterated S-protein. The area of the downfield C-2 peak in the spectrum of 5 days deuterated S-protein is equivalent to 0.35 protons and the area of the upfield peak is equivalent to 0.40 protons. The area of peaks 1, 2, 3 and 4 in the spectrum of 5 days deuterated ribonuclease-A is 0.07, 0.08, 0.35 and 0.26 protons respectively. Thus peak 3, which has an area equivalent to 0.35 protons in the spectrum of 5 days deuterated ribonuclease-A, can be assigned to histidine 119 which has an area equivalent to 0.35 protons in the spectrum of 5 days deuterated S-protein. Peak 2, which has an area equivalent to 0.08 protons in the spectrum of 5 days deuterated ribonuclease-A, is too small to be assigned to histidine 119 and can be assigned to histidine 12. The area of the C-2 peak of histidine 48 is not the same in the spectra of 5 days deuterated ribonuclease-A and S-protein, being equivalent to 0.26 protons in ribonuclease-A spectrum and 0.40 protons in the S-protein spectrum. This discrepancy could be due to the presence of excess noise on the peak due to histidine 48 in the spectrum of the S-protein as this peak appears to have an unsymmetrical shape.

The assignment of peak 2 to histidine 12 and peak 3 to histidine 119 was confirmed by the spectrum obtained for 5 days deuterated S-peptide. S-peptide consists of ribonuclease-A amino acid residues 1-20. Figure 8.7 shows the 100 MHz spectra of the aromatic region of S-peptide, obtained from protonated and 5 days deuterated ribonuclease-A, in 0.10 M sodium acetate, pH 7, at 10^{o}C. The top spectrum is of protonated S-peptide from Meadows et al (1968). An original spectrum of protonated S-peptide was not obtained as the peptide sample was lost when an NMR tube containing the sample broke when the solution froze in the NMR probe at low temperature. There was insufficient time available to prepare another sample. The C-2 peak is very broad (width $\sim$ 15 Hz) as the C-2 proton is involved in a hydrophobic interaction (Meadows et al, 1968). At room temperature the peak is so broad that it can not be seen above the baseline. The area of the peak is equivalent to 0.80 protons.

The spectrum of 5 days deuterated S-peptide shows no sign of a C-2 histidine peak. This is to be expected as the area of peak 2 in the spectrum of 5 days deuterated ribonuclease-A is only equivalent to 0.08 protons. This small area together with the large peak width would result in a peak that is merged into the baseline.

The width of the aromatic peak in the spectrum of the protonated S-peptide is 10 Hz (Meadows et al, 1968) and the width in the deuterated S-peptide spectrum is 14 Hz. This difference could be due to a difference in spectrometer

Figure 8.7


Ribonuclease-S Peptide
0.10 M acetate, pH 7.0
Phenylalanine 8 and
C-4 Histidine 12
Native
(Meadows et al, 1968)
C-2 Histidine 12
400
350
300
250
200
Hz from HOD
Phenylalanine 8 and
C-4 Histidine 12
5 Days deuterated
HOD
Side-band
400
350
300
250
200
Hz from HOD

resolution. It is also noticable that the chemical shift of the aromatic peak is not the same in both spectra. This could be due to the fact that Meadows et al (1968) give the chemical shift in units of Hz from hexamethyldisiloxane (HMS). In changing the chemical shift scale to units of Hz from HOD it has been assumed that HMS resonates at 0δ and HOD at 480 Hz downfield from HMS. As Meadows et al (1968) do not give the full spectrum of the S-peptide, this assumption may not be justified.

8 D DISCUSSION

The results obtained for the exchange rates of the histidine C-2 protons closely follow those that would be expected on the basis of the imidazole group pK's. On the basis of pK alone, it would be expected that histidine 105, with an apparent pK of 6.4, would exchange its C-2 proton for a deuteron at the fastest rate followed by histidine 12, with an apparent pK of 5.8, and histidine 119, with an apparent pK of 5.2, would be expected to have the slowest rate of exchange. As the pK of histidine 48, in D_2O, is not known one cannot forecast a rate of exchange for the C-2 proton.

The experimental results indicate that the C-2 protons of histidine 12 and 105 exchange at about the same rate. As the rate of exchange depends on both the pK of the imidazole group and the second order rate constant, (Chapter 4), it would appear that the second order rate constant for exchange at the C-2 position of histidine 12

is greater than that for exchange at the C-2 position of histidine 105. The value of k_{obs} for exchange at the C-2 position of histidine 48 is intermediate between that for histidine 119 and histidine 12 & 105. This would indicate that histidine 48 has a fairly normal pK ($\sim$6). Meadows et al (1968) have shown that the apparent pK in acetate solution is 6.4. The slower overall exchange rate at the C-2 position of histidine 48 compared with the exchange rate at the C-2 position of histidine 12 & 105 would be due to a smaller value for the second order rate constant caused by histidine 48 being partially buried in the interior of the molecule above pH 5.5 in D_2O solutions.

The NMR spectra obtained on deuterated S-protein and S-peptide show that histidine peaks 2 & 3 may be assigned to histidine 12 and 119 respectively. Thus the assumption of Meadows et al (1968) that peaks 2 & 3 maintain the same relative position in spectra of both ribonuclease-A and S is justified. This work on ribonuclease-A provides additional proof for the assignment given by the above authors.

King & Bradbury (1971) have noted that peak 3, in spectra of ribonuclease-A obtained in D_2O solutions, moves downfield further than peaks 1 and 2 when the pH is reduced below 4. This behaviour suggests the proximity of a carboxyl group, the protonation of which is being reflected in the downfield shift of peak 3. As X-ray crystallographic evidence (Kartha et al, 1967) indicates that aspartic acid 121 is closer to histidine 119 than to histidine 12, this observation provides supporting evidence for the assignment

of peak 3 to histidine 119.

In summary, the assignment of the histidine resonances is as follows. Peak 1 is from histidine 105, peak 2 is from histidine 12, peak 3 is from histidine 119 and peak 4 is from histidine 48. A correct assignment is essential in the development of theories of action of the enzyme based on NMR studies (Ruterjans & Witzel, 1969).

BIBLIOGRAPHY

Aune, K.C. & Timasheff, S.N. (1971) Biochemistry, 10, 1609.

Bak, B., Dambamm, C. & Nicklaisen, F. (1967) Acta. Chem. Scand., 21, 1674.

Bell, F. (1928) J. Chem. Soc., 2776.

Bethune, I.L. & Kegeles, G. (1961) J. Phys. Chem., 65, 1761.

Biltoren, R., Lumry, R., Madison, V. & Parker, H. (1965) Proc. Nat. Acad. Sci. U.S., 54, 1412.

Bloembergen, N., Purcell, E.M. & Pound, R.V. (1948) Phys.Rev. 73, 679.

Bradbury, J.H., Chapman, B.E. & King, N.L. (1971) Inter. J. Protein Res., 3, 351.

Bradbury, J.H. & King, N.L. (1969) Aust. J. Chem., 22, 1083.

Bradbury, J.H. & King, N.L. (1972) Aust. J. Chem., 25, 209.

Bradbury, J.H. & Scheraga, H.A. (1966) J. Amer. Chem. Soc., 88, 4240.

Bradbury, J.H. & Wilairat (1967) Biochem. Biophys. Res. Comm., 29, 84.

Bovey, F.A. (1969) " Nuclear Magnetic Resonance Spectroscopy" Academic Press.

Bovey, F.A., Tiers, G.V. & Filipovich, G. (1959) J. Polymer Sci., 38, 73.

Chervenka, C.H. (1962) J. Biol. Chem., 237, 210.

Crespi, H.L. & Katz, J.J. (1969) Nature, 224, 560.

Crestfield, A.M., Stein, W.H. & Moore, S. (1963) J. Biol. Chem., 238, 618.

Crestfield, A.M., Stein, W.H. & Moore, S. (1963a) J. Biol. Chem., 238, 2413.

Cunningham, L.W., Tietze, F., Green, N.M. & Neurath, H. (1953) Disc. Faraday Soc.,13, 58.

Delaage, M. & Lazdunski, M. (1967) Biochem. Biophys. Res. Comm.,28, 390.

Dayhoff, M.O. (1969) "Atlas of Protein Sequence and Structure" Vol.4, National Biomedical Research Foundation, Maryland.

Dayhoff, M.O. & Eck, R.V. (1968) as above.

Egan, R., Michel, H.O., Schlueter, R. & Jandorf, B.J. (1957) Arch. Biochem. Biophys., 66, 366.

Emsley, J.W., Feeney, J. & Sutcliff, L.H. (1965) "High Resolution Nuclear Magnetic Resonance Spectroscopy" Pergamon Press.

Fersht, A.R. & Requena, Y. (1971) J. Mol. Biol., 60, 279.

Freer, S.T., Kraut, J., Wright, H.T. & Xuong, N.H. (1970) Biochemistry, 9, 1997.

Gladner, J.A. & Neurath, H. (1954) J. Biol. Chem., 206, 911.

Glaskoe, P.K. & Long, F.A. (1960) J. Phys. Chem., 64, 188.

Gutowsky, H.S. & Woessner, D.E. (1966) Phys. Rev., 104, 843.

Harris, J.J. (1956) Nature, 177, 471.

Harris, M. & Randall, J.C. (1965) Chem. Ind. (London), 1728.

Hopkins, T.R. & Spikes, J.D. (1967) Biochem. Biophys. Res. Comm., 28, 480.

Hopkins, T.R. & Spikes, J.D. (1968) ibid 30, 540.

Jansen, E., Nutting, M.D., Jang, R. & Balls, A.K. (1949) J. Biol. Chem., 179, 189.

Jennings, B.R. (1968) Biochem. J., 96, 17.

Hartley, B.S. (1970) Phil. Trans. Royl. Soc. London. B257, 77.

Johnson, C.H. & Knowles, J.R. (1966) Biochem. J., 101, 56.

Josefsson, L. (1958) Ark. Kemi., 12, 183 and 195.

Kartha, G., Bello, J. & Harker, D. (1967) Nature, 213, 826.

Kallos, J & Rizok, D. (1963) J. Mol. Biol., 7, 599.

Kawahara, K. & Tanford, C. (1966) J. Biol. Chem., 241, 3228.

King, N.L. (1970) Ph.D. Thesis, Australian National University.

King, N.L. & Bradbury, J.H. (1971) Nature, 229, 404.

Klotz, I.M. (1970) Arch. Biochem. Biophys., 138, 704.

Kowalsky, A. (1962) J. Biol. Chem., 237, 1807.

Krigbaum, W.R. & Godwin, R.W. (1968) Biochemistry, 7, 3126.

Kubo, R. & Tomita, K. (1954) J. Phys. Soc. Japan, 9, 291.

Kumar, S. & Hem, G.E. (1970) Biochemistry, 9, 291.

Link, T.P. & Stark, G.R. (1968) J. Biol. Chem., 243, 1082.

McDonald, C.C. & Phillips, W.D. (1969) J. Amer. Chem. Soc. 91, 1513.

McDonald, C.C. & Phillips, W.D. (1970) in "Fine Structure of Proteins and Nucleic Acids" Dekker.

Macindoe, H.M. & Reisner, A.H. (1967) Aust. J. Biol. Sci., 20, 141.

Markley, J.L., Putter, I. & Jardetzky, O. (1968) Science, 161, 795.

Martin, C.J. (1962) Biochem. Biophys. Acta., 51, 610.

Martin, C.J. & Frazier, A. (1963) J. Biol. Chem., 238, 3268.

Meadows, D.H., Markley, J.L., Cohn, J.S. & Jardetzky, O. (1967) Proc. Nat. Acad. Sci. U.S., 58, 1307.

Meadows, D.H., Jardetzky, O., Epand, R.M., Ruterjans, H.H. & Scheraga, H.A. (1968) Proc. Nat. Acad. Sci. U.S., 60, 766.

Mildvan, A.S. & Cohn, M. (1970) Adv. in Enzymology, 33, 1.

Morgan, M.S. & Cretcher, L.H. (1948) J. Amer. Chem. Soc. 70, 375.

Moromito, K. & Kegeles, G. (1967) Biochemistry, 6, 3007.

Nakagawa, Y. & Bender, M.L. (1970) Biochemistry, 9, 257.

Neet, K.E. & Brydon, S.E. (1970) Arch. Biochem. Biophys., 136, 223.

Norton, R.S. (1971) private communication.

Olafson, R.A., Thompson, W.R. & Michelman, J.S. (1964) J. Amer. Chem. Soc., 86, 1865.

Oppenheimer, H.L., Labouesse, B. & Hess, G.P. (1966) J. Biol. Chem., 241, 2720.

Perrin, D.D. (1965) "Dissociation Constants of Organic Bases in Aqueous Solution" Butterworths.

Pritchard, A.M. & Richards, R.E. (1966) Trans. Faraday Soc. 62, 1388 and 2014.

Pople, J.A., Schneider, W.G. & Bernstein, H.J. (1959) "High-resolution Nuclear Magnetic Resonance" McGraw-Hill.

Reisner, A.H. & Rowe, J. (1969a) Nature, 222, 558.

Reisner, A.H., Rowe, J. & Macindoe, H.M. (1969b) Biochem. Biophys. Acta., 188, 196.

Reisner, A.H., Rowe, J. & Sleigh, R.W. (1969c) Biochemistry, 8, 4637.

Richards, F.M. & Vithayathil, P.J. (1959) J. Biol. Chem., 234, 1459.

Richards, F.M. & Wyckoff, H.W. (1971) The Enzymes, 4, 647, Academic Press.

Riordan, J.F., Bier, M. & Nord, F.F. (1961) Arch. Biochem. Biophys., 90, 125.

Roa, M.S. & Kegeles, G. (1958) J. Amer. Chem. Soc., 80, 5754.

Roberts, G.C. & Jardetzky, O. (1970) Adv. Protein Chem., 24, 447.

Ruterjans, H.H. & Witzel, H. (1969) Europ. J. Biochem., 9, 118.

Sachs, D.H., Schecter, A.N. & Cohn, J.S. (1971) J. Biol. Chem., 246, 6576.

Sarfare, P.S., Kegeles, G. & Kownrhee, S.J. (1966) Biochemistry, 5, 1389.

Saunders, M., Wishnia, A. & Kirkwood, J.G. (1957) J. Amer. Chem. Soc., 79, 3289.

Saunders, M. & Wishnia, A. (1962) J. Amer. Chem. Soc., 84, 4325.

Schwert, G.W. (1949) J. Biol. Chem., 179, 655.

Schwert, G.W. & Kaufman, A. (1951) J. Biol. Chem., 190, 807.

Simpson, R.B. & Kauzmann, W. (1953) J. Amer. Chem. Soc., 75, 5139.

Smith, E.L. & Brown, D.M. (1952) J. Biol. Chem., 195, 525.

Steiner, R.F. (1954) Arch. Biochem. Biophys., 53, 457.

Staub, H.S. (1964) Tetrahedron Letters, 15, 845.

Tanford, C. (1968) Adv. Protein Chem., 23, 121.

Tinoco, I. (1957) Arch. Biochem. Biophys., 68, 367.

Vaughan, J.D., Mughrabi, Z. & Chung Wu, E. (1970) J. Org. Chem., 35, 1141.

Venkatachalam, C.M. & Ramachandran, G.N. (1969) Ann. Rev. Biochem., 38, 45.

Willumsen, L. (1968) C.R. Trav. Lab. Carlsberg, 36, 327.

Wright, H.T., Kraut, J. & Wilcox, P.E. (1968) J. Mol. Biol., 37, 363.

Wyckoff, H.W., Tsernoglou, D., Hanson, A.W., Knox, J.R., Lee, B. & Richards, F.M. (1970) J. Biol. Chem., 245, 305.

APPENDIX

TREATMENT OF KINETIC RESULTS

The equation for a first order reaction is:

$$\ln c = -k_{obs} t + \ln c_o \qquad (1)$$

where c_o is the concentration of reactant at zero time

t is the time

c is the concentration of reactant at time t

and k_{obs} is the first order rate constant which may be obtained from the slope of the line ln c vs t.

For the histidine C-2 exchange reaction c may be replaced by the proportional area A.

Equation (1) is of the general form

$$y = ax + b \qquad (2)$$

If one has data consisting of n points, the coordinates of which are (x_1y_1, x_2y_2, ...x_ny_n), the line of best fit through these points is obtained by the method of least squares. The line of best fit will have values of

$$a = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2} \qquad (3)$$

$$b = \frac{\sum y - a\sum x}{n} \qquad (4)$$

The standard deviation of the y values from this line is given by:

$$S_y = \sqrt{\frac{n\sum y^2 - (\sum y)^2 - a^2(n\sum x^2 - (\sum x)^2)}{n(n-2)}} \qquad (5)$$

The standard deviation of the slope of the line is given by:

$$S_a = \sqrt{\frac{n}{n\Sigma x^2 - (\Sigma x)^2}\,(S_y)^2} \qquad (6)$$

Values of $k_{obs}/2.303$ were obtained, using equation (3), by substituting $\log_{10}A$ for y and t for x. The value of S_a was also calculated.

The results for a number of compounds, for which the C-2 proton was exchanged at 37°C, are given in the following tables.

T is the time in days, A is the proportional area of the C-2 resonance, L(A) is $\log_{10}$ proportional area and k_{obs} is the pseudo first order rate constant the units of which are $days^{-1}$.

IMIDAZOLE

T	A	L(A)
	pD 3.1	
0.00	1.00	0.00
0.54	1.01	0.01
1.00	1.00	0.00
2.00	1.00	0.00
3.00	1.00	0.00
k_{obs}= 0.00	S_a= 0.00	
	pD 7.0	
0.00	1.00	0.00
0.54	0.90	-0.05
1.00	0.72	-0.14

IMIDAZOLE

T	A	L(A)
	pD 7.0	
2.00	0.59	-0.23
3.00	0.45	-0.36
k_{obs}= 0.28	S_a= 0.01	
	pD 8.8	
0.00	1.00	0.00
0.44	0.62	-0.21
1.00	0.34	-0.47
1.46	0.21	-0.68
2.00	0.14	-0.86
k_{obs}= 1.03	S_a= 0.03	

IMIDAZOLE

T	A	L(A)
	pD 10.4	
0.00	1.00	0.00
0.125	0.90	-0.05
0.21	0.83	-0.08
0.29	0.69	-0.16
0.44	0.58	-0.24
0.54	0.49	-0.31
1.00	0.28	-0.55
1.31	0.23	-0.65

k_{obs}= 1.20 S_a= 0.04

	pD 12.5	
0.00	1.00	0.00
0.125	0.88	-0.06
0.21	0.80	-0.10
0.29	0.66	-0.18
0.44	0.55	-0.26
0.54	0.46	-0.34
1.00	0.27	-0.57
1.31	0.21	-0.68

k_{obs}= 1.23 S_a= 0.05

	pD 13.4	
0.00	1.00	0.00
0.125	0.90	-0.05
0.21	0.86	-0.07
0.29	0.68	-0.17
0.44	0.56	-0.25

IMIDAZOLE

T	A	L(A)
	pD 13.4	
0.54	0.48	-0.32
1.00	0.27	-0.53
1.31	0.23	-0.64

k_{obs}= 1.20 S_a= 0.05

N-ACETYL-L-HISTIDINE

	pD 3.9	
0.00	1.00	0.00
0.125	1.00	0.00
0.292	1.01	0.01
0.46	0.98	-0.01
0.75	1.00	0.00
1.58	1.00	0.00

k_{obs}= 0.00 S_a= 0.01

	pD 6.6	
0.00	1.00	0.00
0.125	0.98	-0.01
0.292	0.94	-0.03
0.46	0.94	-0.03
0.75	0.92	-0.04
1.58	0.78	-0.11

k_{obs}= 0.13 S_a= 0.01

	pD 8.2	
0.00	1.00	0.00
0.125	0.96	-0.02

N-ACETYL-L-HISTIDINE

T	A	L(A)
	pD 8.2	
0.292	0.87	-0.06
0.46	0.83	-0.08
0.75	0.72	-0.14
1.58	0.55	-0.26

k_{obs}= 0.38 S_a= 0.01

	pD 8.9	
0.00	1.00	0.00
0.125	0.93	-0.03
0.292	0.81	-0.09
0.46	0.78	-0.11
0.75	0.66	-0.18
1.58	0.41	-0.39

k_{obs}= 0.56 S_a= 0.01

	pD 10.4	
0.00	1.00	0.00
0.125	0.93	-0.03
0.292	0.81	-0.09
0.46	0.74	-0.13
0.75	0.68	-0.17
1.58	0.37	-0.43

k_{obs}= 0.63 S_a= 0.01

	pD 12.9	
0.00	1.00	0.00
0.125	0.91	-0.04
0.292	0.81	-0.09

N-ACETYL-L-HISTIDINE

T	A	L(A)
0.46	0.73	-0.14
0.75	0.62	-0.21
1.58	0.35	-0.45

k_{obs}= 0.65 S_a= 0.01

L-HISTIDYLGLYCINE

	pD 5.4	
0.00	1.00	0.00
1.00	0.96	-0.02
2.20	1.00	0.00
3.00	0.96	-0.02
4.00	0.96	-0.02
7.00	0.96	-0.02

k_{obs}= 0.01 S_a= 0.01

	pD 7.4	
0.00	1.00	0.00
1.00	0.80	-0.10
2.20	0.66	-0.18
3.00	0.59	-0.23
4.00	0.45	-0.35
7.00	0.26	-0.58

k_{obs}= 0.19 S_a= 0.01

	pD 8.5	
0.00	1.00	0.00
1.00	0.78	-0.11
2.20	0.56	-0.25
3.00	0.48	-0.32

L-HISTIDYLGLYCINE

T	A	L(A)
	pD 8.5	
4.00	0.33	-0.48
7.00	0.16	-0.80
k_{obs}= 0.26	S_a= 0.01	
	pD 9.6	
0.00	1.00	0.00
1.00	0.63	-0.20
2.20	0.30	-0.52
3.00	0.24	-0.63
4.00	0.15	-0.84
- - - - - - -		
7.00	0.07	-1.11
k_{obs}= 0.49	S_a= 0.02	
	pD 11.4	
0.00	1.00	0.00
0.10	0.94	-0.03
0.20	0.90	-0.05
0.30	0.82	-0.09
0.42	0.73	-0.14
1.00	0.53	-0.28
1.25	0.45	-0.35
1.45	0.36	-0.45
k_{obs}= 0.66	S_a= 0.02	

β-ALANYL-L-HISTIDINE

T	A	L(A)
	pD 5.2	
0.00	1.00	0.00
1.00	1.00	0.00
2.00	1.00	0.00
3.00	0.98	-0.01
4.00	1.01	0.01
6.00	1.00	0.00
7.00	1.01	0.01
8.00	0.98	-0.01
10.00	1.00	0.00
k_{obs}= 0.00	S_a= 0.00	
	pD 6.4	
0.00	1.00	0.00
0.396	0.96	-0.02
0.835	0.92	-0.04
1.04	0.92	-0.04
2.44	0.83	-0.08
3.44	0.76	-0.12
4.48	0.71	-0.15
8.30	0.52	-0.28
k_{obs}= 0.08	S_a= 0.00	
	pD 7.6	
0.00	1.00	0.00
1.00	0.68	-0.17
2.00	0.50	-0.30
3.00	0.34	-0.47

β-ALANYL-L-HISTIDINE

T	A	L(A)
	pD 7.6	
4.00	0.25	-0.60
k_{obs}= 0.35	S_a= 0.01	
	pD 8.6	
0.00	1.00	0.00
0.23	0.90	-0.05
0.396	0.84	-0.08
0.835	0.69	-0.16
1.04	0.64	-0.20
2.44	0.32	-0.49
3.44	0.20	-0.70
k_{obs}= 0.47	S_a= 0.01	
	pD 9.0	
0.00	1.00	0.00
0.23	0.91	-0.04
0.396	0.84	-0.08
0.835	0.68	-0.17
1.04	0.62	-0.21
2.44	0.32	-0.49
3.44	0.21	-0.69
k_{obs}= 0.47	S_a= 0.00	
	pD 9.3	
0.00	1.00	0.00
1.00	0.60	-0.22
2.00	0.34	-0.47
3.00	0.22	-0.67

β-ALANYL-L-HISTIDINE

T	A	L(A)
	pD 9.3	
4.00	0.13	-0.88
k_{obs}= 0.51	S_a= 0.01	
	pD 9.9	
0.00	1.00	0.00
0.084	0.96	-0.02
0.23	0.90	-0.05
0.396	0.80	-0.10
0.48	0.73	-0.14
0.835	0.64	-0.20
1.04	0.54	-0.27
1.27	0.48	-0.32
2.44	0.25	-0.60
k_{obs}= 0.57	S_a= 0.01	
	pD 10.4	
0.00	1.00	0.00
0.084	0.91	-0.04
0.23	0.83	-0.08
0.396	0.73	-0.14
0.48	0.72	-0.15
0.835	0.57	-0.24
1.04	0.51	-0.29
1.27	0.44	-0.36
2.44	0.23	-0.64
k_{obs}= 0.63	S_a= 0.02	

β-ALANYL-L-HISTIDINE

pD 12.2

T	A	L(A)
0.00	1.00	0.00
0.084	0.91	-0.04
0.23	0.83	-0.08
0.396	0.74	-0.13
0.48	0.68	-0.17
0.835	0.51	-0.29
1.04	0.44	-0.36
1.27	0.44	-0.36
2.44	0.20	-0.70

k_{obs}= 0.65 S_a= 0.02

pD 13.4

T	A	L(A)
0.00	1.00	0.00
0.084	0.96	-0.02
0.23	0.82	-0.09
0.396	0.76	-0.12
0.48	0.66	-0.18
0.835	0.64	-0.20
1.04	0.50	-0.30
1.27	0.46	-0.34
2.44	0.20	-0.70

k_{obs}= 0.65 S_a= 0.01

L-HISTIDINE

pD 1.2

T	A	L(A)
0.00	1.00	0.00
1.00	0.97	-0.02
2.00	1.00	0.00
3.00	1.00	0.00
4.00	1.00	0.00
7.00	1.02	0.01
10.00	1.00	0.00
15.00	1.04	0.05

k_{obs}= -0.01 S_a= 0.01

pD 5.5

0.00	1.00	0.00
1.00	0.98	-0.01
2.00	0.93	-0.03
3.00	0.91	-0.04
4.00	0.88	-0.06
7.00	0.81	-0.09
14.00	0.65	-0.19
18.00	0.58	-0.24
21.00	0.56	-0.25

k_{obs}= 0.03 S_a= 0.01

pD 6.8

0.00	1.00	0.00
0.95	0.90	-0.05
1.95	0.78	-0.11
2.95	0.66	-0.18

L-HISTIDINE

T	A	L(A)
	pD 6.8	
3.75	0.38	-0.22
k_{obs}= 0.14	S_a= 0.01	
	pD 7.2	
0.00	1.00	0.00
1.00	0.86	-0.07
2.00	0.70	-0.17
3.00	0.58	-0.24
4.00	0.46	-0.34
7.00	0.36	-0.45
k_{obs}= 0.16	S_a= 0.01	
	pD 7.4	
0.00	1.00	0.00
0.31	0.94	-0.03
1.06	0.78	-0.11
2.12	0.58	-0.24
3.18	0.46	-0.34
3.94	0.38	-0.42
k_{obs}= 0.25	S_a= 0.01	
	pD 7.9	
0.00	1.00	0.00
0.96	0.75	-0.12
1.96	0.60	-0.23
2.96	0.40	-0.40
4.00	0.32	-0.49
k_{obs}= 0.29	S_a= 0.02	

L-HISTIDINE

T	A	L(A)
	pD 8.2	
0.00	1.00	0.00
0.31	0.92	-0.04
1.06	0.76	-0.12
2.12	0.56	-0.25
3.18	0.43	-0.37
3.94	0.35	-0.46
k_{obs}= 0.27	S_a= 0.00	
	pD 8.6	
0.00	1.00	0.00
0.31	0.90	-0.05
1.06	0.70	-0.16
2.12	0.50	-0.30
3.18	0.43	-0.37
3.94	0.33	-0.48
k_{obs}= 0.29	S_a= 0.02	
	pD 9.4	
0.00	1.00	0.00
1.00	0.70	-0.16
2.00	0.48	-0.32
3.00	0.31	-0.51
4.00	0.20	-0.70
7.00	0.11	-0.96
k_{obs}= 0.32	S_a= 0.02	
	pD 9.5	
0.00	1.00	0.00

L-HISTIDINE

T	A	L(A)
	pD 9.5	
1.00	0.66	-0.18
2.00	0.50	-0.30
3.00	0.32	-0.49
4.00	0.19	-0.72

k_{obs}= 0.40 S_a= 0.02

	pD 9.9	
0.00	1.00	0.00
0.104	0.96	-0.02
0.208	0.87	-0.06
0.312	0.87	-0.06
0.44	0.74	-0.13
0.52	0.72	-0.14
1.00	0.58	-0.24
1.12	0.53	-0.28
1.44	0.45	-0.35

k_{obs}= 0.56 S_a= 0.02

	pD 10.9	
0.00	1.00	0.00
0.104	0.94	-0.03
0.208	0.87	-0.06
0.312	0.80	-0.10
0.44	0.74	-0.13
0.52	0.70	-0.16
1.00	0.49	-0.31
1.12	0.48	-0.32

L-HISTIDINE

T	A	L(A)
	pD 10.9	
1.44	0.36	-0.44

k_{obs}= 0.71 S_a= 0.01

	pD 11.4	
0.00	1.00	0.00
0.042	0.96	-0.02
0.084	0.91	-0.04
0.166	0.91	-0.04
0.50	0.73	-0.14
1.00	0.53	-0.28
2.40	0.21	-0.68
3.00	0.14	-0.85
3.25	0.13	-0.89
4.42	0.12	-0.92
7.20	0.08	-1.10

k_{obs}= 0.65 S_a= 0.01

	pD 12.4	
0.00	1.00	0.00
0.042	1.00	0.00
0.084	0.96	-0.02
0.166	0.90	-0.05
0.290	0.80	-0.10
0.875	0.56	-0.25
1.00	0.48	-0.32
1.17	0.44	-0.36
1.95	0.26	-0.59

L-HISTIDINE

T	A	L(A)
	pD 12.4	
3.87	0.11	-0.95
4.92	0.09	-1.03
6.92	0.08	-1.10

k_{obs}= 0.69 S_a= 0.02

	pD 12.9	
0.00	1.00	0.00
0.042	0.96	-0.02
0.084	0.96	-0.02
0.180	0.86	-0.07
0.500	0.68	-0.17
1.00	0.48	-0.32
2.60	0.16	-0.80
- - - - - -		
3.80	0.10	-1.00

k_{obs}= 0.70 S_a= 0.02

L-HISTIDINE

T	A	L(A)
	pD 13.4	
0.00	1.00	0.00
0.104	0.96	-0.02
0.208	0.87	-0.06
0.398	0.78	-0.11
0.480	0.70	-0.15
0.960	0.54	-0.27
1.170	0.46	-0.34
1.390	0.37	-0.43

k_{obs}= 0.69 S_a= 0.02

Values below the dashed line were not used in the calculations.

PUBLICATIONS

The following publication was derived from work described in this thesis:-

Bradbury, J.H., Chapman, B.E. & King, N.L. (1971) Inter. J. Protein Res., 3, 351.

APPENDIX II

A ERRATA

Acknowledgements, para. 2, line 2: replace "Deptartment" by "Department".
Page 3, para. 2, line 12: replace "reviewd" by "reviewed".
Page 11, para. 2, line 3: delete "of".
Page 12, para. 4, line 3: replace "useing" by "using".
Page 17, para. 3, line 3: replace "DCL" by "DCl".
Page 39, para. 2, line 13: replace "occuring" by "occurring".
Page 45, para. 1, line 8: replace "explaination" by "explanation".
Page 45, para. 1, line 10: replace "hign" by "high".
Page 56, para. 1, line 4: replace "decending" by "descending".
Page 57, para. 1, line 5: replace "Unfortunatly" by "Unfortunately".
Page 75, para. 3, line 6: replace "explaination" by "explanation".
Page 81, para. 3, line 1: replace "has" by "have".
Page 81, para. 2 of footnote, line 1: replace "codeing" by "coding".
Page 89, line 2: replace "thiol" by "disulphide".

B COMMENTS ON THE SPECIFICITY OF CHEMICAL MODIFICATION REACTIONS ON ∝- CHYMOTRYPSIN AND RIBONUCLEASE-A.

Diisopropylphosphate-serine 195-∝-chymotrypsin.

The reaction of diisopropylfluorophosphate with ∝-chymotrypsin is specific for serine 195. The inhibitor will not react with the free amino acid or with denatured ∝-chymotrypsin (Dixon and Webb, 1966). The special reactivity of serine 195 is due to the spatial arrangement of other nearby amino acid residues. A one to one mole ratio of inhibitor to enzyme will completely inactivate the enzyme.

p-fluorophenylsulphonyl-serine 195-∝-chymotrypsin.

The reaction of aromatic sulphonyl chlorides with ∝-chymotrypsin is specific for serine

195. A one to one mole ratio of inhibitor to enzyme is sufficient to inactivate the enzyme and the inhibitor will not react with denatured ∝-chymotrypsin (Kallos and Rizok, 1963).

3-N-methyl-histidine 57-∝-chymotrypsin.

Nakagawa and Bender (1970) have shown that the reaction between ^{14}C-methyl p-nitrobenzenesulphonate and ∝-chymotrypsin occurs mainly at histidine-57. The inhibitor will react at a very slow rate (1/30,000 of the rate for histidine-57) with free histidine amino acid. High voltage electrophoresis of the enzymic hydrolysis products of modified ∝-chymotrypsin showed that most of the radioactivity was associated with histidine 57 and a slight amount with histidine 40. The reaction with histidine 40 is thought to occur by the same non-specific method as for the free amino acid. The rapid reaction with histidine 57 is due to the orientation of the inhibitor in the enzyme active site in such a way as to lower the activation energy for the reaction.

S-methyl-methionine 29-ribonuclease-A.

Link and Stark (1968) have studied the specificity of the reaction between ^{14}C-methyl iodide and ribonuclease-A. An analysis of peptide fragments, obtained from enzymic and chemical cleavage of modified ribonuclease-A, showed that 92% of the ribonuclease molecules were methylated on methionine 29. The other methionine residues, 13, 30 and 79, were methylated to a minor extent. Methionine 29 is partially exposed at the surface of the ribonuclease molecule while the other methionine residues are buried inside the molecule.

C DISCUSSION OF MECHANISMS OF DEUTERATION OF IMIDAZOLE AND HISTIDINE DERIVATIVES.

Although the mechanism of Vaughan et al (1970) has been selected for the rate-determining step in the deuteration of imidazole and histidine derivatives, there is another possible mechanism that would satisfy the pD-dependence of the observed rate.

This mechanism would involve a reaction between the uncharged form of the imidazole ring and the solvent and can be represented by the following equation:

$$\text{Rate} = k_{obs}\left[\text{Imid}^t\right] = k_1\left[D_2O\right]\left[\text{Imid}\right]$$

where k_{obs} is the observed pseudo-first order rate constant, $\left[\text{Imid}^t\right]$ is the total concentration of imidazole rings, k_1 is the second order rate constant, $\left[D_2O\right]$ is the concentration of solvent and $\left[\text{Imid}\right]$ is the concentration of uncharged imidazole rings. As the relationship between pD and concentration of uncharged imidazole rings is sigmoidal, the relationship between pD and rate must be sigmoidal as k_1 and $\left[D_2O\right]$ are constant.

The second inflection observed in the pD-rate curve for the exchange of the histidine C-2 proton would have to be explained by postulating that the second order rate constant k_1, for the deuteration of histidine species 2 (page 32 of text), would have to be greater than for species 1 as the concentration of uncharged imidazole rings falls when the ∝-amino proton is removed.

Leach (1972) has suggested that the two possible mechanisms might be resolved by conducting the reaction in an equimolar mixture of D_2O/H_2O. If the uncharged imidazole ring mechanism is correct the rate of deuteration will be halved as the concentration of D_2O is halved. If a ylide is being formed its rate of formation will be unchanged by the solvent composition. The rate of formation of the ylide, however, cannot be measured directly. Its rate of formation is measured by the subsequent reaction of ylide with D_2O. In an equimolar mixture of D_2O/H_2O half the ylide would react with D_2O to give the deuterated product and half would react with H_2O to give the protonated product. Thus the observable rate of reaction would be reduced by half.

Although the uncharged imidazole ring mechanism could be used to explain the pD-rate profile it was rejected for the following reasons.

1. Olafson et al (1964) observed that N,N-dialkylimidazolium salts, which are permanently in the cationic form, underwent rapid exchange at the C-2 carbon in D_2O. The half-life of the reaction of the dimethylimidazolium ion at pD 8.9, 31°C is 4.5 minutes. This observation strongly implicates the charged form of the imidazole ring as the reactive species.

2. Vaughan et al (1970) exchanged the C-2 proton of imidazole at 65°C and observed that the proton was exchanged at a constant rate, equal to 10% of the limiting rate at high pD, between pD 0-4. This observation can be explained by postulating that, at this high temperature, the charged form of imidazole can react with D_2O, a weak base, to give the ylide. The concentration of the charged form of imidazole is effectively constant between pD 0 and 4. The concentration of the uncharged form of imidazole, although very small, increases tenfold with each increasing pD unit in this pD range. Thus if the uncharged form of imidazole is the reactive species one would expect to see a logarithmic increase in rate of deuteration with increasing pD rather than the observed straight line with a slope of zero.

3. If the uncharged imidazole ring mechanism is adopted, the second inflection observed in the pD-rate profile for the deuteration of histidine can only be explained by an increase in the second order rate constant for histidine species 2 over that for species 1. This would imply that the lability of the C-2 proton is increased when the α-amino proton is removed. This is the direct opposite of what would be expected as the presence of a nearby positive charge would increase the lability of the C-2 proton while its removal would decrease the lability of the proton.

D THE DIFFERENTIAL DEUTERATION OF HISTIDINE DERIVATIVES.

The main aim of the work on the exchange of the C-2 proton in histidine model compounds was to see if conditions could be found where different histidine derivatives could be deuterated at different rates. These same conditions would then be applied to try to differentially deuterate histidine residues in ribonuclease-A.

Figure 4.6 (b) shows that, at pD 8.5, there is a linear relationship between k_{obs} and pK for a number of histidine derivatives. This result is fortuitous, as the rate of deuteration depends on both the pK of the imidazole ring and the second order rate constant (page 37 of text), and does not occur at any other pD. For example at high pD the rate of C-2 proton exchange is the same for all four histidine derivatives. Imidazole has not been included on the graph as it is not a histidine derivative. The value of k_{obs} at pD 8.5, for imidazole, is about 1.0. This is higher than that observed for N-acetylhistidine which has the same pK as imidazole. This is due to the higher second order rate constant for imidazole (page 36 of text).

Although the charged form of imidazole and histidine species l^{+} both have an overall charge of +1 the rate of deuteration for imidazole is greater than that for histidine. This is due to the difference in the pK of the imidazole rings as histidine has a pK of 6.6 and imidazole 7.6. The positive and negative charges on the main chain of histidine do not have an equal and opposite effect on the pK of the imidazole ring as their distances from the ring are not the same. Bradbury and Scheraga (1966) have shown that removal of the positive charge raises the pK of the imidazole ring by one pH unit while removal of the negative charge lowers the pK by one tenth of a pH unit.

BIBLIOGRAPHY

Dixon, M. and Webb, E. C. (1966) "Enzymes", page 473, Longmans.

Leach, S. J. private communication.